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Award Number: DAMD17-99-1-9133

TITLE: Eosinophils as Mediators of DNA Oxidative Damage in Breast Cancer

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REPORT DATE: July 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2001	Annual (1 Jul 00 - 30 Jun 01)

4. TITLE AND SUBTITLE
Eosinophils as Mediators of DNA Oxidative Damage in Breast Cancer

5. FUNDING NUMBERS
DAMD17-99-1-9133

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The overall goal of this proposal is to test the hypothesis that eosinophils promote DNA oxidative damage in breast carcinoma. DNA oxidative damage is linked to mutation, transformation and cancer development and eosinophil peroxidase (EPO), a hemoprotein secreted from eosinophils, is present in the majority of breast cancer biopsies. Our initial aim was to determine whether EPO promotes oxidative damage of cellular DNA through formation of mutagenic hydroxyl radical ($^{\bullet}$OH)-generated bases. Last year we published a study (Biochemistry, (2000) 39:5474) that demonstrated activated leukocytes can oxidatively damage DNA, RNA and the nucleotide pool through halide-dependent formation of $^{\bullet}$OH. $^{\bullet}$OH-dependent damage of DNA was quantified by monitoring the content of 8-hydroxyguanine (8OHG), an established $^{\bullet}$OH-specific DNA oxidation product that is mutagenic and implicated in breast cancer development and progression to metastatic disease. To test the hypothesis that EPO promotes DNA oxidative damage in human breast carcinoma, we have identified a family of novel brominated DNA oxidation products. These may serve as "molecular fingerprints" for DNA damage by the EPO pathway of eosinophils. The results of our initial studies identifying brominated bases by mass spectrometry were recently published (Biochemistry, (2001) 40:2041-2051). We are presently performing studies aimed at quantifying these EPO-specific brominated bases in a well-characterized repository of breast carcinoma and microscopically normal breast tissue specimens.

14. SUBJECT TERMS	15. NUMBER OF PAGES
breast cancer, eosinophil, oxidation, free radical, DNA damage	32
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Cover..... 1

SF 298..... 2

Table of Contents..... 3

Introduction..... 4

Body..... 4-6

Key Research Accomplishments..... 6

Reportable Outcomes..... 6

Conclusions..... 7,8

References..... 8

Appendices.....

Introduction

The contribution of oxidative processes to DNA damage and carcinogenesis is now widely accepted. However, the precise chemical pathways involved remain unclear. One method that has contributed significantly to the conclusion that DNA undergoes oxidative damage is the detection of multiple distinct stable markers of free-radical reactions *in vivo*. For example, markers of $^{\bullet}OH$-, reactive nitrogen species- and aldehyde-dependent damage of DNA have been used to implicate specific chemical mechanisms of DNA damage *in vivo*. Eosinophil recruitment is characteristic of many cancers and numerous chronic parasitic infections are associated with a dramatic increase in risk for development of cancer, yet the potential role of eosinophils in mediating DNA damage and cancer development has not received attention. In our proposal we hypothesized that eosinophils may contribute to DNA oxidative damage and cancer. The vast majority of breast cancers stain positively for eosinophil peroxidase (EPO), a hemoprotein specific for eosinophils. EPO catalyzes oxidation reactions as part of its normal role in host defenses. We hypothesize that the presence of EPO in breast cancer tissues suggests that EPO could contribute to oxidative modification of DNA and the nucleotide pool, and thus potentially play a role in the disease process. We proposed to identify chemical mechanisms through which eosinophils might contribute to DNA oxidative damage. We also proposed to define novel specific chemical markers indicative of eosinophil-mediated DNA damage in vivo. Finally, we proposed to use mass spectrometry to establish whether these markers are enriched in breast cancer specimens - thus identifying for the first time a pathogenic role for eosinophils in cancer development.

Body

Task 1. To determine whether eosinophil peroxidase promotes oxidative damage of cellular DNA through formation of mutagenic hydroxyl radical-generated bases.

All of the sub-goals as outlined in Task 1 of the approved Statement of Work were achieved in last years (1999-2000) reporting interval. Detailed descriptions of the methodology and results were provided with the preceding annual report. A copy of a publication that is based upon this work is provided as Appendix item 1:

Z. Shen, W. Wu and S. L. Hazen "Activated leukocytes oxidatively damage DNA, RNA and the nucleotide pool through halide-dependent formation of hydroxyl radical. *Biochemistry* (2000), 39:5474-5482.

Task 2. To test the hypothesis that eosinophil peroxidase promotes DNA oxidative damage in human breast carcinoma

a. *Synthesize, HPLC purify and confirm structures of brominated bases by ESI/MS, GC/MS and multinuclear NMR* - Done, see below

b. *Synthesize, HPLC purify and confirm structure of stable isotope-labeled brominated bases by GC/MS* - Done, see below

c. *optimize DNA hydrolysis/digestion/sample work-up for quantification of brominated bases in DNA exposed to HOBr* - Done, see below

d. *perform experiments to evaluate Br-dG and Br-dC formation in calf thymus DNA exposed to HOBr, and then the EPO-H_2O_2-Br- system* - Done, see below

We have completed all of the above sub-goals as outlined in Task 1 of the approved Statement of Work. Detailed descriptions of the methodology and results are enumerated in a recent publication that is based upon this work. A copy of the manuscript is provided as Appendix item 2:

Shen, Z., Mitra, S.N., Wu, W., Chen, Y., Yang, Y., Qin, J and Hazen, S.L. "Eosinophil peroxidase brominates free nucleotides and double stranded DNA," *Biochemistry* (2001) 40:2041-2051.

The results of these studies were also presented at 2 international meetings. A copy of the abstracts are provided as Appendix items 3 and 4:

a) Shen, Z., and Hazen, S.L. "Eosinophil peroxidase brominates free nucleotides and double stranded DNA," Abstract 263, of 7th Annual Meeting of the Oxygen Society, Nov 16-20, 2000. *Free Radical Biology & Medicine*, (2000) Vol 29, Supplement I, S85.

b) Shen, Z., and Hazen, S.L. "Oxidative damage of DNA by peroxidase-mediated bromination," Oral Presentation, June (2000), American Society of Mass Spectrometry Meeting, Long Beach, CA.

Briefly, we show that EPO effectively uses plasma levels of bromide as co-substrate to brominate bases in nucleotides and double-stranded DNA forming several stable novel brominated adducts. Products were characterized by HPLC with on-line UV spectroscopy and electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS). Ring assignments for brominated purine bases as their 8-bromo adducts were identified by NMR spectroscopy. Using stable isotope dilution LC/ESI/MS/MS, we show that while guanine is the preferred purine targeted for bromination as a free nucleobase, 8-bromoadenine is the major purine oxidation product generated following exposure of double-stranded DNA to either HOBr or the EPO-H_2O_2-Br^- system. Bromination of nucleobases was inhibited by scavengers of hypohalous acids like the thioether methionine, but not by large a molar excess of primary amines. Subsequently, *N*-mono-bromoamines were demonstrated to be effective brominating agents for both free nucleobases and adenine within intact DNA. A rationale for selective modification of adenine, but not guanine, in double-stranded DNA based upon stereochemical criteria is presented. Collectively, these results suggest that specific brominated DNA bases may serve as novel markers for monitoring oxidative damage of DNA and the nucleotide pool by brominating oxidants.

Work still in progress:

e. perform experiments to assess ability of EPO to promote Br-dG and Br-dC formation in cell culture model as well as the factors influencing DNA damage by this pathway

We are in the process of performing studies with cultured cells (HA1 cells) exposed to the EPO-H_2O_2-Br^- system of activate eosinophils. The in vitro models we are employing are the same as those developed and utilized for demonstration of $^{\bullet}OH$ formation by EPO, as assessed by 8OHG generation in cytosolic bases and DNA of target cells (*Biochem.* (2000), 39:5474-5482).

f. quantify brominated bases (Br-dG and Br-dC) in DNA recovered from normal, dysplastic, cancerous and metastatic breast tissue

This work is still in progress. We have observed that some additional methodological issues are required for handling of breast biopsy tissues compared to *in vitro* studies with cultured cells and isolated chromatin as the target. The high fat content of the sample makes the overall recovery of the analytes of interest poor using our routine preparation techniques, interfering with mass spectrometric detection and analysis and limiting our sensitivity of detection. We are in the process of working out new sample preparation methods that include organic extraction of lipids prior to analysis. We then will apply the stable isotope dilution LC/ESI/MS/MS methods developed to analyze breast biopsy specimens from normal and cancerous specimens.

Thus far, the results of studies related to this proposal are consistent with the hypotheses we aim to test in this proposal - that eosinophils may contribute - rather than protect from - the development of cancer via DNA oxidative damage. Studies with isolated enzyme (EPO) and isolated eosinophils all demonstrate that oxidative modification of DNA can occur through EPO-dependent pathways. At the present time there are no suggested changes in the Statement of Work or the Aims as outlined in the original proposal.

Key Research Accomplishments

1) The leukocyte peroxidases EPO and MPO generate halogenating oxidants that combine with superoxide to form hydroxyl radical like species capable of damaging DNA.

2) Exposure of cells to an extracellular source of peroxidase-generated reactive halogen promotes hydroxylation of DNA, RNA and the nucleotide pool in the presence of superoxide. This results in formation of mutagenic bases and DNA damage characteristic of that observed in breast cancer and progression to metastatic disease.

3) Exposure of free nucleotides and DNA to either HOBr or the EPO-H2O2-Br- system results in bromination of DNA bases. Several distinct brominated bases are known to be mutagenic.

4) The structures of the halogenated nucleobases have been defined.

5) Stable isotope dilution mass spectrometry-based methods have been developed to quantify the content of these novel molecular markers.

6) Insights into the chemical mechanisms accounting for nucleotide bromination and the structural/steric requirements for bromination of free nucleobases vs double stranded DNA have been defined.

Reportable outcomes

Manuscripts (Items 1 and 2 of Appendix) :

Shen, Z., Wu,. and Hazen, S.L. "Activated leukocytes oxidatively damage DNA, RNA and the nucleotide pool through halide-dependent formation of hydroxyl radical. *Biochemistry* (2000), 39:5474-5482.

Shen, Z., Mitra, S.N., Wu, W., Chen, Y., Yang, Y., Qin, J and Hazen, S.L. "Eosinophil peroxidase brominates free nucleotides and double stranded DNA," *Biochemistry* (2001) 40:2041-2051.

Abstracts and presentations (Appendix Items 3 and 4):

Shen, Z., and Hazen, S.L. "Eosinophil peroxidase brominates free nucleotides and double stranded DNA," Abstract 263, of 7th Annual Meeting of the Oxygen Society, Nov 16-20, 2000. *Free Radical Biology & Medicine*, (2000) Vol 29, Supplement I, S85.

Shen, Z., and Hazen, S.L. "Oxidative damage of DNA by peroxidase-mediated bromination," Oral Presentation, June (2000), American Society of Mass Spectrometry Meeting, Long Beach, CA. #755.

patents - None

degrees obtained - None

development of tissue repositories/cell lines - None

informatics - None

funding applied for based upon work - None

employment/research opportunities applied for/received - None

Conclusions

The contribution of oxidative processes to carcinogenesis is now widely accepted. Much progress in this area involves use of stable markers of free-radical reactions to identify specific chemical mechanisms of DNA damage *in vivo*. For example, hydroxylated, nitrated, aldehyde-modified, and chlorinated bases have been characterized and used to determine mechanisms of DNA, RNA and nucleotide damage *in vitro* and *in vivo* (*48-55*). However, the potential role of brominating oxidants in DNA damage and cancer development has not received much attention. The results of the present studies suggest that formation of reactive brominating species by the EPO-H_2O_2-Br^- system of eosinophils may be one pathway these cells contribute to oxidative modification of DNA and the nucleotide pool. Recent studies identify brominating oxidants as a distinct class of oxidants formed following eosinophil activation *in vivo*. Moreover, numerous cancers are notable for a significant eosinophilic infiltration in the cancerous tissues. The specific brominated bases identified may thus serve as markers for future studies aimed at determining the potential role of brominating oxidants in DNA oxidative damage *in vivo*.

Based upon results from the present report and recent published studies, we have generated a model of potential pathways through which brominating oxidants may contribute to oxidative modification of free bases, RNA and DNA. Upon activation, the NADPH oxidase complex of eosinophils forms $O_2^{\bullet-}$, which both spontaneously and enzymatically dismutates to form H_2O_2. Concomitantly, eosinophil activation leads to the secretion of EPO into the extracellular compartment. In the presence of plasma levels of Br^-, EPO generates brominating oxidants like HOBr, which can directly brominate free nucleotides and DNA forming stable mono-brominated adducts. Identification of brominated adducts of each free base was confirmed by HPLC with on-line ESI-MS analysis. In the case of brominated purine bases, structural characterization as the 8-bromo-substituted analogs of adenine and guanine was further confirmed by NMR and tandem mass spectrometry. EPO-generated HOBr can also react with $O_2^{\bullet-}$ to form a $^{\bullet}OH$-like oxidant. The content of 8-hydroxyguanine, a marker of $^{\bullet}OH$-dependent DNA damage, was recently shown to significantly increase in DNA, RNA and the nucleotide pool of cells exposed to a hypohalous acid generating system and enhanced intracellular $O_2^{\bullet-}$.

One interesting result in the present study is the observation that 8-bromoadenine is a major stable purine oxidation product formed following exposure of double-stranded DNA to the EPO-H_2O_2-Br^- system, while no detectable 8-bromo adduct for guanine in DNA was formed. These results are the reverse of the rank order for bromination observed with free nucleosides at neutral pH. Conformational analysis of the steric requirements for incorporation of a halide into the C-8 position of purine bases in both free nucleosides and within the major groove of the DNA double helix provides a rational for these observations. Addition of a bulky bromo group at the C-8 position ortho to the glycosyl link, due to steric constraints, forces the purine bases to adopt a *syn* glycosyl conformation. In contrast, a smaller hydroxyl group at the C-8 position of purines, such as in formation of 8-hydroxyguanine or 8-hydroxyadenine, is readily accommodated in the *anti* glycosyl conformation. While a *syn* conformation for purine bases are quite frequent in free nucleosides, a change in the glycosyl torsion from *anti* to *syn* in double-stranded DNA would result in a significant free energy expenditure due to changes in the base pairing scheme (below). For purine bases either free or in the C2'-endo sugar pucker (B-DNA), a *syn* glycosyl conformation results in an additional stability contributed by an intramolecular O5'-H···N3 hydrogen bond. Therefore, bromination at C-8 in the free purine bases or the nucleosides can occur readily.

In contrast, as noted above, for bromination to occur at the C-8 position of either adenine or guanine in a DNA double helix, the purine base must convert its glycosyl conformation from *anti* to *syn*. In both cases, this conversion will necessitate the transformation of a Watson-Crick base pair to a Hoogsteen pair. A Hoogsteen A•T base pair, like its Watson-Crick pair, has two hydrogen bonds and can be formed without loss of stability. For a G•C Hoogsteen pair to be formed with the G in *syn* conformation, the complementary cytosine base needs to be protonated. This will impose a significant free energy restriction by the demand of a lower pKa for such a transition to occur. In addition, in the Hoogsteen geometry a G•C pair loses stability by having only two hydrogen bonds, in contrast to three hydrogen bonds for its Watson-Crick geometry. These restrictions readily explain the predominance of 8-BrA formation and absence of any 8-BrG adducts, following exposure of calf-thymus DNA to either reagent HOBr or the EPO-H_2O_2-Br^- system.

One critical question that has not yet been resolved is whether bromination of purine and pyrimidine targets takes place *in vivo*. This is the focus of our research efforts currently. Our goal is to analyze breast cancer specimens. However, we may also look at other sites of inflammation where eosinophil-mediated inflammation and cancer development occur. For example, we can get access to biopsy specimens from individuals with schistosomiasis, a chronic parasitic infection that results in dramatic increases in cancer. Detection of brominated DNA and/or nucleotides from isolated schistosomes would serve as in vivo "proof of concept" for the central idea of the proposal. Clearly, the probability of a brominating oxidant diffusing through a gauntlet of cytosolic scavengers unscathed before reaching a nuclear DNA base as its ultimate target will be a low probability event (as are all DNA oxidation events). However, it should also be recognized that the oxidation event does not have to take place inside the cell nucleus, but may occur either within the cytosol (i.e. the nucleotide pool) or even within the extracellular compartment. Parasitic infections are accompanied by increased cell death and lysis at the site of inflammation. Moreover, brominated bases can be taken up and incorporated into DNA and RNA of cultured mammalian cells. Though mammalian cells are equipped with numerous surveillance mechanisms for removal of modified bases from the nucleotide pool, the fidelity of these systems is not absolute. Indeed, exposing cultured cells to brominated bases in the media results in sister chromatid exchanges and mutation. We have recently demonstrated that bromination of extracellular targets (protein tyrosine residues) by activated eosinophils occurs *in vivo* at sites of inflammation. The ability of certain free nucleobases to undergo bromination at neutral pH suggests that similar events may occur *in vivo*. Thus, in the setting of a chronic parasitic infection where decades of eosinophil-mediated inflammatory injury can occur, bromination of extracellular or cytosolic nucleobases may occur.

In summary, the present results suggest that specific brominated DNA bases may serve as novel and specific markers for monitoring oxidative damage of DNA and the nucleotide pool by brominating oxidants. Moreover, they identify adenine, rather than guanine, as a more likely target for halogenation of purine bases within double-helix DNA. The detection of brominated bases in eosinophil-rich inflammatory lesions or cancers would strongly suggest that brominating oxidants formed by these cells contribute to the development of DNA damage in these disorders.

References - None

Appendix

Item 1 - Shen, Z., Wu,. and Hazen, S.L. "Activated leukocytes oxidatively damage DNA, RNA and the nucleotide pool through halide-dependent formation of hydroxyl radical. *Biochemistry* (2000), 39:5474-5482.

Item 2 - Shen, Z., Mitra, S.N., Wu, W., Chen, Y., Yang, Y., Qin, J and Hazen, S.L. "Eosinophil peroxidase brominates free nucleotides and double stranded DNA," *Biochemistry* (2001) 40:2041-2051.

Abstracts and presentations (Appendix Items 3 and 4):

Item 3 - Shen, Z., and Hazen, S.L. "Eosinophil peroxidase brominates free nucleotides and double stranded DNA," Abstract 263, of 7th Annual Meeting of the Oxygen Society, Nov 16-20, 2000. *Free Radical Biology & Medicine*, (2000) Vol 29, Supplement I, S85.

Item 4 - Shen, Z., and Hazen, S.L. "Oxidative damage of DNA by peroxidase-mediated bromination," Oral Presentation, June (2000), American Society of Mass Spectrometry Meeting, Long Beach, CA. #755.

Activated Leukocytes Oxidatively Damage DNA, RNA, and the Nucleotide Pool through Halide-Dependent Formation of Hydroxyl Radical

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Biochemistry®

Reprinted from
Volume 39, Number 18, Pages 5474–5482

Activated Leukocytes Oxidatively Damage DNA, RNA, and the Nucleotide Pool through Halide-Dependent Formation of Hydroxyl Radical†

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Received December 7, 1999

ABSTRACT: A variety of chronic inflammatory conditions are associated with an increased risk for the development of cancer. Because of the numerous links between DNA oxidative damage and carcinogenesis, a potential role for leukocyte-generated oxidants in these processes has been suggested. In the present study, we demonstrate a novel free transition metal ion-independent mechanism for hydroxyl radical (•OH)-mediated damage of cellular DNA, RNA, and cytosolic nucleotides by activated neutrophils and eosinophils. The mechanism involves reaction of peroxidase-generated hypohalous acid (HOCl or HOBr) with intracellular superoxide ($O_2^{\bullet-}$) forming •OH, a reactive oxidant species implicated in carcinogenesis. Incubation of DNA with either isolated myeloperoxidase (MPO) or eosinophil peroxidase (EPO), plasma levels of halides (Cl^- and Br^-), and a cell-free $O_2^{\bullet-}$ -generating system resulted in DNA oxidative damage. Formation of 8-hydroxyguanine (8-OHG), a mutagenic base which is a marker for •OH-mediated DNA damage, required peroxidase and halides and occurred in the presence of transition metal chelators (DTPA ± desferrioxamine), and was inhibited by catalase, superoxide dismutase (SOD), and scavengers of hypohalous acids. Similarly, exposure of DNA to either neutrophils or eosinophils activated in media containing metal ion chelators resulted in 8-OHG formation through a pathway that was blocked by peroxidase inhibitors, hypohalous acid scavengers, and catalytically active (but not heat-inactivated) catalase and SOD. Formation of 8-OHG in target cells (HA1 fibroblasts) occurred in all guanyl nucleotide-containing pools examined following exposure to both a low continuous flux of HOCl (at sublethal doses, as assessed by [^{14}C]adenine release and clonogenic survival), and hyperoxia (to enhance intracellular $O_2^{\bullet-}$ levels). Mitochondrial DNA, poly A RNA, and the cytosolic nucleotide pool were the primary targets for oxidation. Moreover, modest but statistically significant increases in the 8-OHG content of nuclear DNA were also noted. These results suggest that the peroxidase−H_2O_2−halide system of leukocytes is a potential mechanism contributing to the well-established link between chronic inflammation, DNA damage, and cancer development.

The contribution of oxidative processes to carcinogenesis is now widely accepted (*1*−*7*). Much progress in this area involves use of stable markers of free-radical reactions to identify specific chemical mechanisms of DNA damage in vivo (*8*−*11*). DNA damage caused by the highly reactive hydroxyl radical (•OH)[1] has been linked to neoplasia in a number of biological systems (*1*−*12*).

DNA damage by •OH generates characteristic mutagenic base lesions, such as 8-hydroxyguanine (8-OHG) (*8*−*12*). Significant progressive increases in the content of •OH-generated lesions in the DNA from normal, premalignant, cancerous, and metastatic tissues have been demonstrated utilizing a variety of independent analytical methods (*5*−*7, 12, 13*). The direct correlation observed between free radical-mediated 8-OHG formation and carcinogenesis has been attributed to the ability of this modified base to induce mutagenic events during DNA synthesis (*14, 15*). The pathways responsible for generating •OH in vivo are unclear. Free metal ions, like Fe^{2+} or Cu^{2+}, are thought to play a role because of their well-known ability to catalyze conversion of reduced oxygen species such as superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) into the more reactive •OH (eq 1) (*16*):

$$O_2^{\bullet-} + H_2O_2 \xrightarrow{M^{2+}} {}^{\bullet}OH + OH^- + O_2 \qquad (1)$$

Numerous sources of $O_2^{\bullet-}$ and H_2O_2 during carcinogenesis have been suggested, including redox cycling of estrogens (*17, 18*), xenoestrogens (*19, 20*), and xenobiotics such as polychlorinated biphenyls (*21*), peroxidase-oxidized products (*22, 23*), and a variety of intracellular sources (*24, 25*). The

† This work was supported in part by a grant from the Department of Defense (BC980289) and by grants from the National Institutes of Health (HL62526 and HL29582). Z.S. is a recipient of a Jane Coffin Childs Memorial Fund for Medical Research Fellowship.

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[1] Abbreviations: 8-OHG, 8-hydroxyguanine; Atz, aminotriazole; BHT, butylated hydroxytoluene; DTPA, diethylenetriaminepentaacetic acid; EPO, eosinophil peroxidase; G, guanine; HBSS, Hanks balanced salt solution; HOCl, hypochlorous acid; HOBr, hypobromous acid; H_2O_2, hydrogen peroxide; HPLC-EC, high-performance liquid chromatography with electrochemical detection; MPO, myeloperoxidase; $O_2^{\bullet-}$, superoxide anion; •OH, hydroxyl radical; PMA, phorbol myristate acetate; SOD, superoxide dismutase.

10.1021/bi992809y CCC: $19.00
Published on Web 04/14/2000

mechanism(s) responsible for generating •OH during the development of cancer and metastases formation in vivo is (are) still unclear.

Phagocytic cells such as neutrophils and eosinophils are a particularly attractive source of reactive intermediates since they have evolved enzymatic mechanisms to generate an arsenal of reactive species as part of their normal function. Neutrophils and eosinophils play an essential role in tissue surveillance in host defense mechanisms. These cells inflict oxidative damage upon invading parasites and pathogens; however, the reactive species they form can also damage normal tissues (*26*−*30*). Activated neutrophils and eosinophils employ NADPH oxidase to catalyze conversion of molecular oxygen to $O_2^{\bullet-}$, which dismutates to form H_2O_2 (*31*). Research thus far into the mechanisms of phagocyte-dependent DNA damage has primarily focused on the potential role(s) of $O_2^{\bullet-}$ and H_2O_2 in DNA damage through metal ion-dependent generation of •OH, or in the oxidative conversion of precarcinogens into carcinogens (*4, 32*−*37*). In addition to its ability to reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) (the presumed catalyst involved in formation of •OH), a role for $O_2^{\bullet-}$ in releasing iron from protein-bound iron−sulfur clusters has also been suggested (*38*).

An alternative pathway for •OH formation in vivo may involve myeloperoxidase (MPO) and eosinophil peroxidase (EPO), abundant heme proteins secreted by neutrophils and eosinophils, respectively. MPO and EPO amplify the oxidative potential of H_2O_2 by generating reactive halogen (*39*−*43*), reactive nitrogen (*44*−*49*), reactive aldehyde (*28, 50, 51*), and diffusible radical species (*45, 52, 53*). Under physiological concentrations of halides, major initial oxidants formed by MPO and EPO are hypochlorous acid (HOCl) and hypobromous acid (HOBr), respectively. Studies with spin trapping agents (*54, 55*) and chemical traps of •OH (*56, 57*) have demonstrated that hypohalous acids can generate •OH following reaction with $O_2^{\bullet-}$ (eq 2):

$$O_2^{\bullet-} + HOX \rightarrow {}^{\bullet}OH + X^- + O_2 \ (X = Cl \text{ or } Br) \quad (2)$$

This reaction is analogous to the Haber−Weiss reaction (eq 1) where H_2O_2 is replaced by HOCl or HOBr. In the absence of metal ions, eq 2 is at least 6 orders of magnitude faster than the Haber−Weiss reaction (*57*−*59*).

In the present study, we examine the potential role of leukocyte-generated hypohalous acids and intracellular $O_2^{\bullet-}$ as mediators of •OH-dependent damage of cellular DNA, RNA, and free nucleotides. We now demonstrate that exposure of DNA to either isolated MPO or EPO and a $O_2^{\bullet-}$-generating system results in •OH-mediated DNA damage through a free metal ion-independent, halide-dependent pathway. We also demonstrate that activated neutrophils and eosinophils oxidatively damage DNA through this pathway. Finally, we demonstrate that exposure of target cells to both an exogenous source of hypohalous acids and enhanced intracellular $O_2^{\bullet-}$ production results in significant •OH-mediated damage of nuclear DNA, mitochondrial DNA, RNA, and cytosolic nucleotides.

EXPERIMENTAL PROCEDURES

Materials

Organic solvents (HPLC-grade) and H_2O_2 (30%; ACS grade) were obtained from Fisher Chemical Co. (Pittsburgh, PA). 2-Deoxy-8-hydroxyguanosine (8-OHdG) was obtained from Sigma Chemical Co. (St. Louis, MO). Chelex-100 resin (200−400 mesh, sodium form) was obtained from BioRad (Hercules, CA). 8-Hydroxyguanine (8-OHG) and 8-[1,2-^{3}H]-OHG were prepared as described (*9*). DNA Extractor WB kits were obtained from Wako Chemical (Richmond, VA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

Methods

General Procedures. Homogeneous human MPO and porcine EPO were isolated and quantified as described (*43*). Rat liver DNA was extracted and isolated as described (*60*). Neutrophils and eosinophils were isolated by buoyant density centrifugation (*61*). Low levels of contaminating eosinophils or neutrophils in neutrophil and eosinophil preparations, respectively, were then removed by cell sorting (*62*). Buffers were prepared with distilled deionized water. To remove any remaining potential adventitious metals that could participate in the generation of •OH, all reagents, enzymes, and buffers were treated with chelex-100 resin, and reactions were performed in the presence of the metal chelator diethylenetriaminepentaacetic acid (DTPA). All reaction vials, pipet tips, and pH electrodes were rinsed with 100 μM DTPA (pH 7.0) prior to use.

DNA Modification. A stock solution of calf thymus DNA (1 mg/mL) in 20 mM sodium phosphate buffer (pH 7.0) was treated with chelex-100 resin and centrifuged to remove resin, and the pH was adjusted to 7.0 using chelex-treated monobasic sodium phosphate buffer (20 mM) prior to use. If not used immediately, stock solutions were sparged and stored under argon in gastight brown glass vials. Typically, calf thymus DNA (0.8 mg/mL final) was incubated with chelex-treated MPO or EPO (100 nM), a cell-free superoxide generating system comprised of lumazine (0.4 mM), and chelex-treated bovine milk xanthine oxidase (XO, 80 milliunits/mL; Boehringer Mannheim) in the presence or absence of halides. Mixtures were incubated at 37 °C for 1 h, and the reaction was stopped by extraction of DNA using the NaI chaotropic method (*60*).

DNA modification by isolated human neutrophils or human eosinophils was performed by incubating freshly isolated chelex-treated rat liver DNA (0.8 mg/mL final) with cells (5×10^5 cells/mL) in chelex-treated Hank's balanced salt solution (HBSS) supplemented with DTPA (pH 7.4) and plasma levels of Br^- (i.e., 100 μM NaBr). Leukocytes were stimulated with 200 nM phorbol myristate acetate (PMA). In some cases, the suspensions also contained one of the following: 500 units/mL chelex-treated bovine liver catalase, 30 units/mL chelex-treated superoxide dismutase (SOD), 1 mM azide, 10 mM aminotriazole (ATZ), or 100 μM desferrioxamine. Following incubation, cells were pelleted, and the DNA in supernatants was extracted and analyzed for 8-OHG content.

DNA modification of target cells was done using Chinese hamster fibroblasts (HA1 cell line). HA1 cells were maintained in Eagle's minimum essential medium supplemented with fetal calf serum and penicillin−streptomycin (100 units/mL−0.1 mg/mL) in a humidified 5% CO_2 and air environment. Cells were placed in HBSS supplemented with DTPA (100 μM) during the 4 h treatments. The exogenous HOCl-

generating system used was comprised of MPO (100 nM), glucose (in media)/glucose oxidase (10 ng/mL), and Cl^- (in media). Under these conditions, a continuous flux of HOCl of 0.08 μM/min is produced, as assessed by the taurine monochloramine method (*63*), in HBSS supplemented with 10 mM taurine. In some experiments, HA1 cells were maintained in an incubator at an oxygen concentration to 95% as the 4 h hyperoxia condition as described (*64*). Following the 4 h reaction period at 37 °C, cells from multiple dishes were recovered, pooled and pelleted, and washed with ice-cold HBSS supplemented with 100 μM DTPA and butylated hydroxytoluene (BHT, 50 μM), and the content of 8-OHG in at least 20 μg of nuclear DNA, mitochondrial DNA, poly A RNA, and cytosolic nucleotides was then determined by reverse-phase HPLC-EC.

HA1 cellular homogenate was generated in chelex-treated sodium phosphate buffer (50 mM, pH 7.0) supplemented with DTPA (100 μM) and BHT (50 μM). Nuclei and mitochondria were isolated by conventional subcellular fractionation methods prior to isolation of DNA by the NaI chaotropic method (*60*). RNA was isolated as described by Fiala (*65*) with the addition of BHT (100 μM) and desferol (100 μM) to all solutions. Protein in cytosol (100,000*g* supernatant of homogenate) was precipitated by trichloroacetic acid (30% v/v, 0 °C). The samples were spun at 2000*g*, and the resultant supernatant was passed through a DTPA (100 μM)-rinsed 3000 kDa molecular mass cutoff filter and used for analysis of 8-OHG content in the cytosolic free pool. DNA, RNA, and the low molecular weight cytosolic fraction were alternately vacuum-degassed and purged with argon 5 times. Samples were then incubated with formic acid (60% v/v) at 130 °C for 45 min under a blanket of argon. Hydrolysates were resuspended in 200 μL of chelex-treated H_2O and subjected to HPLC-EC analysis.

Reverse Phase HPLC-EC Quantification of 8-OHG. The contents of 8-OHG and guanine (G) in hydrolysates were determined by reverse phase HPLC-EC using an ESA (Cambridge, MA) CoulArray HPLC instrument equipped with 8 electrochemical cells (channels) arranged in series and set to increasing specified potentials as follows: channel 1 (100 mV); channel 2 (250 mV); channel 3 (290 mV); channel 4 (350 mV); channel 5 (480 mV); channel 6 (580 mV); channel 7 (640 mV); and channel 8 connected to a variable UV−Vis detector. Samples were injected onto a Progel TSK ODS-AD TM column (5 μm, 4.6 × 250 mm) equilibrated with mobile phase A (15 mM lithium phosphate, 3 mg/L lithium dodecyl sulfate, pH 3.2). Products were eluted at a flow rate of 1 mL/min with a nonlinear gradient generated with mobile phase B (50% methanol, 15 mM lithium phosphate 3 mg/L lithium dodecyl sulfate, pH 3.2) as follows: isocratic solution at 0% mobile phase B for 30 min, 0−25% mobile phase B over 60 min, 25−100% mobile phase B for 2 min, and isocratic elution at 100% mobile phase B for 20 min. In preliminary studies, peak identity as 8-OHG was established by reverse phase HPLC with on-line electrospray mass spectrometry. Recoveries were also established by spiking samples with high specific activity ^{3}H-labeled 8-OHG standard and then collecting the final peak of interest and counting recovered mass with a scintillation counter. Peak identity was subsequently routinely established by demonstrating the appropriate retention time, redox potential, and ratio of integrated currents in adjacent channels. The content of guanine (G) was determined by UV monitoring at 256 nm. Authentic 8-OHG and G standards were also used to generate external calibration curves.

Measurement of Cellular Injury and Clonogenic Survival Experiments. The degree of cellular injury was measured by the specific release of ^{14}C from proliferating cells previously loaded with ^{14}C-labeled adenine as described (*66*). Clonogenic survival experiments were performed in 60 mm dishes and grown exponentially for 2 days, at which time the cell density was approximately 5 × 10^4/cm^2 as described (*64*). During survival experiments, cultures were treated for 4 h with 4 mL of the exposure indicated in Table 1. Cultures were then washed 3 times with sterile Puck's saline and trypsin-treated, and the resulting single-cell suspension was counted (by Coulter counter). Suspensions were serially diluted and replicates plated for colony survival. After 8−10 days incubation at 37 °C, colonies were fixed in 70% ethanol, stained with Crystal Violet, and counted under a dissecting microscope. Plating efficiencies were approximately 70−85% for all untreated cells.

Statistical Analysis. Differences between treated groups were determined using the paired Student's *t* test assuming a normal distribution. Significance levels were set at $\alpha = 0.05$ for two-tailed tests. When multiple comparisons were made, a Bonferoni correction to the significance criterion for each test was made.

RESULTS

MPO and EPO Promote •OH-Mediated Damage of DNA. Calf thymus DNA was incubated in the presence of a cell-free $O_2^{\bullet-}$-generating system (lumazine/xanthine oxidase) in chelex-100-treated sodium phosphate buffer (pH 7.0) supplemented with the metal chelator DTPA and plasma levels of halides (100 mM Cl^- and 100 μM Br^-). DNA in the reaction mixture was then precipitated and hydrolyzed, and the content of 8-OHG was determined by HPLC-EC as described under Experimental Procedures. In the absence of the leukocyte peroxidases, small but detectable peaks with identical retention time and electrochemical potential to those of authentic 8-OHG were observed (Figure 1, "− MPO" and "− EPO" chromatograms). The modest levels of 8-OHG present under these conditions were similar to those observed in untreated DNA (Figure 2) and were consistent with background levels of the adduct in commercial preparations of calf thymus DNA, as previously reported (*60, 67*). Upon addition of either purified chelex-treated MPO or EPO to the reaction mixtures, significant levels of the •OH-dependent product 8-OHG were formed (Figure 1, "+ MPO" and "+ EPO" chromatograms, and Figure 2). Importantly, formation of the mutagenic base upon addition of either MPO or EPO was halide-dependent (Figure 2), consistent with •OH formation by interaction of $O_2^{\bullet-}$ and a peroxidase-generated hypohalous acid (eq 2). For example, in mixtures containing isolated MPO, the content of 8-OHG formed was dramatically attenuated by omission of Cl^-, a major substrate of MPO (*39*), despite the presence of physiological levels of Br^-. Likewise, omission of Br^-, a preferred substrate of EPO (*42*), from DNA mixtures containing EPO, a $O_2^{\bullet-}$-generating system, and plasma levels of Cl^-, resulted in a significant reduction in the extent of 8-OHG formed (Figure 2). Finally, omission of xanthine oxidase from reaction mixtures ablated 8-OHG formation (Figure 2).

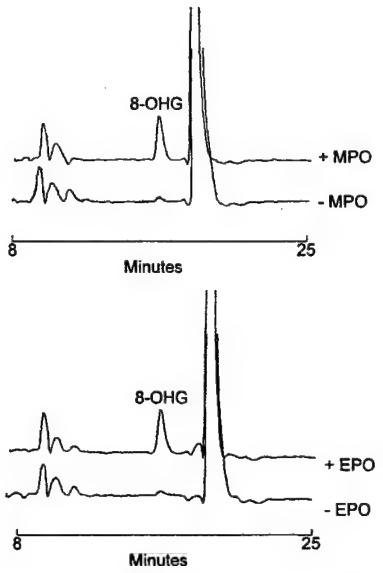

FIGURE 1: Reverse phase HPLC-EC detection of 8-OHG in DNA exposed to the MPO (top panel) and EPO (bottom panel) systems. Calf thymus DNA (400 μg, 0.8 mg/mL final) was incubated for 3 h at 37 °C in sodium phosphate buffer (20 mM, pH 7.0) supplemented with DTPA (100 μM), NaCl (100 mM), NaBr (100 μM), and a cell-free $O_2^{\bullet-}$ generating system (0.4 mM lumazine + 80 milliunits/mL xanthine oxidase) in the absence ("− MPO" and "− EPO") or presence ("+ MPO" and "+ EPO") of either MPO or EPO (105 nM each). DNA was then precipitated, washed, hydrolyzed, and then analyzed by reverse phase HPLC-EC as described under Experimental Procedures. Full scale = 50 nA. EPO, eosinophil peroxidase; MPO, myeloperoxidase; 8-OHG, 8-hydroxyguanine.

Mechanism of 8-OHG Formation during Exposure of DNA to MPO and EPO. To further explore the mechanism of DNA damage by the $^{\bullet}OH$-like oxidant formed by MPO and EPO, we first evaluated the halide concentration-dependence of 8-OHG formation. Each isolated peroxidase was individually incubated with DNA, a cell-free $O_2^{\bullet-}$ -generating system, and differing concentrations of either Cl^- or Br^- in chelex-100-treated buffer supplemented with DTPA, and the 8-OHG content of DNA was then determined by HPLC-EC analysis. Dose-dependent formation of 8-OHG by physiologically relevant levels of Cl^- and Br^- in reaction mixtures containing MPO and EPO, respectively, was observed (Figure 3). Moreover, addition of desferrioxamine, a chelator which potently blocks iron ion-dependent formation of $^{\bullet}OH$, did not completely block 8-OHG formation. In contrast, 8-OHG production was inhibited by addition of methionine, a scavenger of hypohalous acids (*63*), as well as either catalase or SOD, scavengers of H_2O_2 and $O_2^{\bullet-}$, respectively (Figure 3). The present results thus distinguish $^{\bullet}OH$-dependent DNA damage mediated by MPO and EPO from classic free transition metal ion-catalyzed Fenton and Haber Weiss reactions; rather than being inhibited by metal chelators, peroxidase-dependent formation of $^{\bullet}OH$ likely occurs through secondary oxidation reactions between a hypohalous acid and $O_2^{\bullet-}$.

Activated Neutrophils and Eosinophils Promote $^{\bullet}OH$-Mediated DNA Damage through Interaction of a Peroxidase-Generated Hypohalous Acid and $O_2^{\bullet-}$. Exposure of calf thymus DNA to either activated neutrophils or eosinophils in chelex-100-treated media supplemented with DTPA resulted in modest increases in 8-OHG (data not shown).

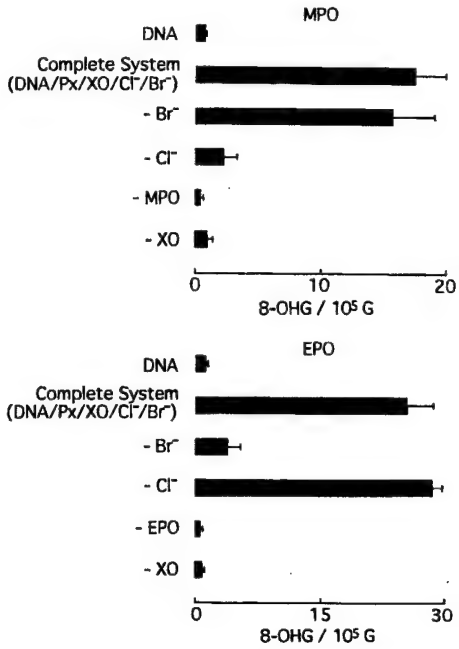

FIGURE 2: Reaction requirements for 8-OHG formation in DNA exposed to MPO (top panel) and EPO (bottom panel). Calf thymus DNA (400 μg, 0.8 mg/mL final) was incubated for 4 h at 37 °C in sodium phosphate buffer (20 mM, pH 7.0) supplemented with DTPA (100 μM), NaCl (100 mM), NaBr (100 μM), and a cell-free $O_2^{\bullet-}$ generating system (0.4 mM lumazine + 80 milliunits/mL xanthine oxidase) in the presence of 100 nM MPO (top panel, Complete System) or EPO (bottom panel, Complete System). Additions or deletions to the Complete Systems were as indicated. Following reaction, DNA was precipitated, washed, and hydrolyzed, and the content of 8-OHG present was determined by HPLC-EC as described under Experimental Procedures. Data represent the mean ± SD of triplicate determinations. EPO, eosinophil peroxidase; G, guanine; MPO, myeloperoxidase; 8-OHG, 8-hydroxyguanine; Px, peroxidase; XO, xanthine oxidase.

However, these increases were difficult to detect reproducibly because of the relatively high background level of 8-OHG in commercial preparations of calf thymus DNA. We therefore subsequently used freshly isolated rat liver DNA as a target for cell-dependent oxidation reactions, since it possessed at least 10-fold lower background levels of 8-OHG. Incubation of rat liver DNA with either PMA-stimulated neutrophils or eosinophils generated significant levels of 8-OHG (Figure 4). 8-OHG formation required cell activation, was only modestly attenuated by addition of desferrioxamine, and was blocked by addition of peroxidase inhibitors (aminotriazole or NaCN) and hypohalous acid scavengers (methionine) (Figure 4). Both SOD and catalase, but neither heat-inactivated SOD nor heat-inactivated catalase, dramatically inhibited 8-OHG production, indicating that both $O_2^{\bullet-}$ and H_2O_2 were required for leukocyte-dependent 8-OHG formation under these conditions. Collectively, these results are consistent with a role for $O_2^{\bullet-}$ and a peroxidase−H_2O_2−halide system—not a free transition metal ion-dependent pathway—in promoting 8-OHG generation by the leukocytes.

Formation of Intracellular 8-OHG following Exposure of Cells to a Nonlethal Flux of HOCl. Experiments thus far described were designed to identify a free metal ion-independent mechanism of 8-OHG formation in isolated DNA exposed to either purified peroxidase−H_2O_2−halide systems or isolated activated human leukocytes. To further assess the potential pathophysiological significance of this

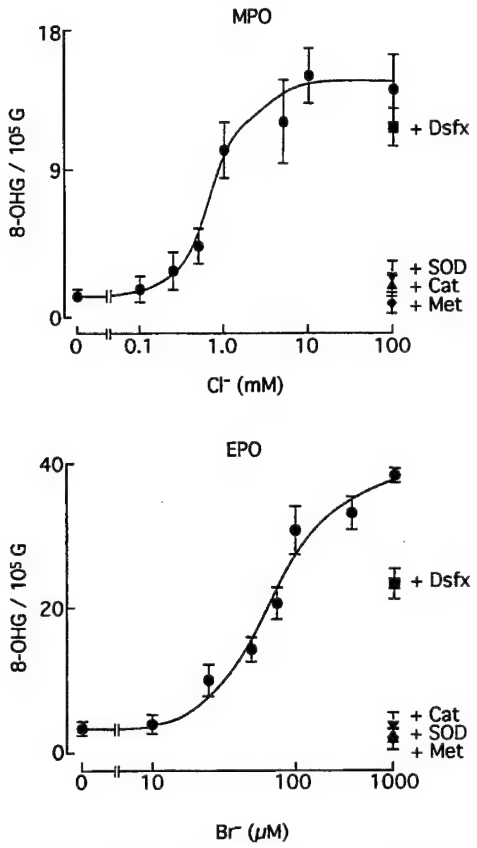

FIGURE 3: Halide concentration dependence of 8-OHG formation by MPO (top panel) and EPO (bottom panel). (Top panel) Calf thymus DNA (400 μg, 0.8 mg/mL final) was incubated for 4 h at 37 °C in sodium phosphate buffer (20 mM, pH 7.0) supplemented with DTPA (100 μM), 100 nM MPO, a cell-free $O_2^{\bullet-}$ generating system (0.4 mM lumazine + 80 milliunits/mL xanthine oxidase), and the indicated concentration of NaCl. In parallel (bottom panel), calf thymus DNA (400 μg, 0.8 mg/mL final) was also incubated for 4 h at 37 °C in sodium phosphate buffer (20 mM, pH 7.0) supplemented with DTPA (100 μM), 100 mM NaCl, 100 nM EPO, a cell-free $O_2^{\bullet-}$ generating system (0.4 mM lumazine + 80 milliunits/mL xanthine oxidase), and the indicated concentration of NaBr. The content of 8-OHG in DNA was then determined by reverse phase HPLC-EC as described under Experimental Procedures. Where indicated, desferrioxamine (100 μM), SOD (30 units/mL), bovine liver catalase (500 units/mL), or methionine (100 μM) were included during reactions. Data represent the mean ± SD of triplicate determinations. Cat, catalase; Dsfx, desferrioxamine; SOD, superoxide dismutase; Met, methionine.

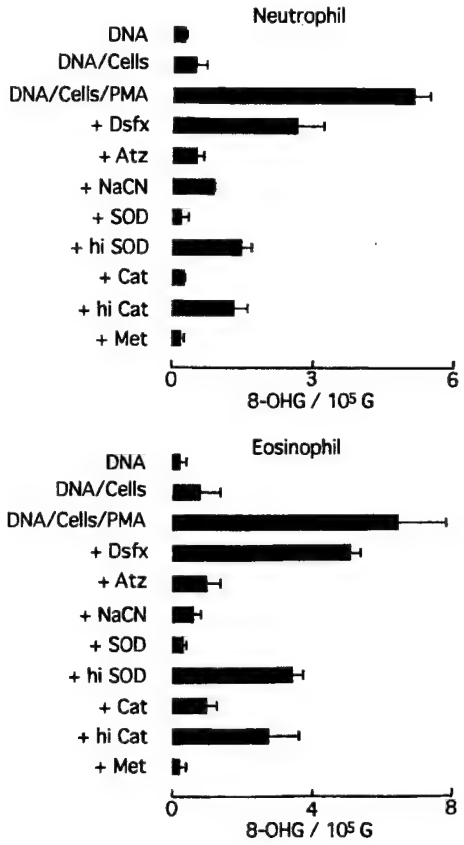

FIGURE 4: 8-OHG formation in rat liver DNA by stimulated neutrophils (top panel) and eosinophils (bottom panel). Rat liver DNA (400 μg, 0.8 mg/mL final) was incubated with the indicated additions in HBSS (pH 7.4) supplemented with DTPA (100 μM) and NaBr (100 μM) for 90 min at 37 °C. Where indicated, (top panel) neutrophils or (bottom panel) eosinophils (5 × 10^5 cells/mL) were included. In some cases, the suspensions also contained phorbol myristate acetate (200 nM), bovine liver catalase (500 units/mL), superoxide dismutase (30 units/mL), NaCN (1 mM), aminotriazole (10 mM), or desferrioxamine (100 μM). Following incubation, cells were pelleted, and DNA in supernatants was recovered and analyzed for 8-OHG content by HPLC-EC as described under Experimental Procedures. Data represent the mean ± SD of triplicate determinations. Atz, 3-aminotriazole; Cat, catalase; hi Cat, heat-inactivated catalase; hi SOD, heat-inactivated superoxide dismutase; Met, methionine; PMA, phorbol myristate acetate; SOD, superoxide dismutase.

pathway for DNA damage, we examined whether intracellular 8-OHG could be formed following exposure of target cells to a sublethal flux of hypohalous acids. Such conditions are likely to mimic those present in vivo at sites of chronic inflammation. HA1 fibroblasts incubated under control conditions (4 h in chelex-100-treated HBSS supplemented with 100 μM DTPA under 5% CO_2, 95% air) contained low but detectable levels of 8-OHG in nuclear DNA (Table 1) at levels comparable to those reported in other cells (*60*). Increased levels of 8-OHG (relative to nuclear DNA) were observed in mitochondrial DNA, and to a lesser degree poly A RNA and the cytosolic free nucleotide pool, of control cells (Table 1). These results are consistent with studies identifying a high steady-state oxidation level in mitochondrial DNA relative to DNA from the protected environment of the nucleus (*68*). Exposure of HA1 cells to a low continuous flux of HOCl for 4 h resulted in a significant increase in the 8-OHG content of mitochondrial DNA (Table 1). These results suggest that exogenous HOCl may interact with $O_2^{\bullet-}$ generated by mitochondria to promote $^{\bullet}OH$-dependent damage of mitochondrial DNA.

To further explore the potential role of DNA damage by hypohalous acid-generated $^{\bullet}OH$, we examined conditions where intracellular production of $O_2^{\bullet-}$ might be increased. Hyperoxia was chosen because it is a nonpharmacological method of enhancing intracellular production of $O_2^{\bullet-}$ (*69*). Brief exposure (4 h) of HA1 cells to hyperoxic conditions resulted in no significant increase in 8-OHG content. However, cells exposed to a brief period of both hyperoxia and the HOCl-generating system resulted in marked increases in the 8-OHG content of mitochondrial DNA, poly A RNA, and cytosolic nucleotides (Table 1). Small but statistically significant increases in the level of 8-OHG in nuclear DNA were noted as well (Table 1). The mechanism of intracellular 8-OHG formation in HA1 cells was consistent with the requirement for HOCl generation by the MPO−H_2O_2−Cl^- system since omission of either MPO or glucose oxidase from the culture medium resulted in a marked decrease in

Table 1: Intracellular 8-OHG Formation after Brief Exposure to HOCl and Hyperoxia[a]

exposure (4 h)	poly A RNA (8-OHG/10^5 G)	mitochondrial DNA (8-OHG/10^5 G)	cytosolic pool (8-OHG/10^5 G)	nuclear DNA (8-OHG/10^5 G)	specific [^{14}C]adenine release (%)	clonogenic survival[d] (%)
control[b]	1.3 ± 0.9	5.2 ± 1.1	1.0 ± 0.9	0.33 ± 0.07	0	100
(A) MPO/GOx	1.4 ± 0.2	7.2 ± 1.4**	1.4 ± 0.2	0.40 ± 0.14	26	85 ± 3
(B) hyperoxia[c]	2.0 ± 0.4	6.9 ± 2.5	1.6 ± 0.4	0.31 ± 0.04	<5	98 ± 5
(A + B) complete	7.2 ± 3.0***	75.3 ± 6.2***	8.1 ± 1.3***	0.57 ± 0.22*	38	82 ± 4
−MPO	2.7 ± 1.8	7.6 ± 3.7	1.7 ± 0.8	0.41 ± 0.14	7	96 ± 5
−GOx	0.8 ± 0.3	5.9 ± 0.9	0.8 ± 0.3	0.31 ± 0.13	<5	99 ± 4

[a] Chinese hamster fibroblasts (HA1 cell line) were incubated for 4 h at 37 °C in 5% CO_2, 95% air in media alone (control); in the presence of an exogenous HOCl-generating system (condition A); in 95% O_2, 5% CO_2 (condition B); in the presence of both a HOCl-generating system and hyperoxia (complete system, conditions A + B); or under conditions of the complete system in the absence of either MPO or glucose oxidase (GOx) as described under Experimental Procedures. Cells were then recovered, and the 8-OHG contents of nuclear DNA, mitochondrial DNA, poly A RNA, and the cytosolic pool were determined as described under Experimental Procedures. In parallel incubations, the specific release of [^{14}C]adenine and clonogenic cell survival assays were performed as described under Experimental Procedures. Data represent the mean ± SD of triplicate determinations. *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$ vs control. [b] 5% CO_2 and 95% air. [c] 5% CO_2 and 95% O_2. [d] Normalized to plating efficiency of 76%.

the 8-OHG content of DNA, RNA, and cytosolic nucleotides (Table 1). A mitochondrial source of $O_2^{\bullet-}$ generation during hyperoxia seems likely given the markedly enhanced levels of 8-OHG in mitochondrial DNA noted.

Hypohalous acids are potent cytotoxins (*26*). Moreover, exposure of HA1 cells to prolonged (>40 h) hyperoxia is known to promote cell death (*64*). It was therefore important to establish that the brief exposure to hyperoxia and low levels of HOCl used did not result in significant toxicity and cell lysis. Initial studies examining lactate dehydrogenase (LDH) release into media were abandoned because control studies demonstrated that HOCl can inhibit LDH activity. We therefore screened for cell toxicity (and membrane leak) by quantifying the specific release of radioactivity from HA1 cells preloaded with ^{14}C-labeled adenine (*66*). In parallel, we examined the mitotic competence of treated cells using clonogenic cell survival assays (*64*). Under the conditions employed, brief exposure of HA1 cells to hyperoxia failed to result in any detectable injury, as assessed by either specific [^{14}C]adenine release or clonogenic cell survival (Table 1). Exposure of cells (4 h) to the low flux of HOCl employed (Table 1, condition "A") resulted in 26% specific [^{14}C]adenine release while 85% of cells remained clonogenically competent. HA1 cells exposed to both hyperoxia and the exogenous HOCl-generating system under conditions identical to those which produced significant levels of intracellular 8-OHG (Table 1, conditions "A + B") resulted in release of 38% of preloaded [^{14}C]adenine while demonstrating an overall clonogenic survival of 82%. These results demonstrate that the brief duration of hyperoxia and modest levels of HOCl employed are not lethal to the majority of cells. They also suggest that a significant portion of HA1 cells initially injured possess adequate repair mechanisms to survive and subsequently undergo mitosis.

DISCUSSION

In this study we investigated the ability of peroxidase−H_2O_2 systems of leukocytes to promote DNA oxidative damage through halide-dependent formation of $^{\bullet}$OH. Multiple lines of evidence support a role for peroxidase-generated hypohalous acids and $O_2^{\bullet-}$ in promoting $^{\bullet}$OH production (eq 2) and subsequent DNA damage. In the absence of free transition metal ions, DNA incubated in the presence of a $O_2^{\bullet-}$ generating system was not significantly modified, as assessed by nominal levels of 8-OHG (Figure 1). However, when isolated MPO or EPO was added to reaction mixtures, 8-OHG was readily detected (Figure 1). Thus, formation of the $^{\bullet}$OH-generated product was dependent on the presence of a peroxidase. A critical role for hypohalous acid generation was confirmed to be a necessary intermediate in 8-OHG formation by demonstrating an absolute halide-dependence for the reaction (Figures 2 and 3). Physiological levels of chloride and bromide were utilized by MPO and EPO, respectively, for generating the $^{\bullet}$OH DNA adduct (Figure 3). Moreover, the requirement for peroxidase-generated hypohalous acid and $O_2^{\bullet-}$ in DNA modification by leukocytes activated in the presence of transition metal ion chelators was apparent by demonstrating that 8-OHG formation by neutrophils and eosinophils was attenuated in the presence of peroxidase inhibitors (e.g., aminotriazole, cyanide), hypohalous acid scavengers (e.g., methionine), and catalytically active SOD. The inhibitory activity of catalytically active catalase is attributable to its ability to block hypohalous acid formation by the peroxidase−H_2O_2−halide system of leukocytes. Finally, it should be noted that all of the reactions in the present study were performed in the presence of the chelator DTPA and used buffers, reagents, and enzymes which were chelex-treated to avoid any potential adventitious transition metal ion-catalyzed formation of $^{\bullet}$OH. Taken together, these results demonstrate that one potential mechanism for generating $^{\bullet}$OH and promoting nucleotide and DNA damage is through reaction of $O_2^{\bullet-}$ with leukocyte-generated hypohalous acids.

Substantial evidence exists linking inflammation, phagocyte-generated oxidants, and peroxidases to carcinogenesis. Clinical studies have documented the association between inflammation and cancer for decades (*1−7, 70, 71*). However, the reaction mechanisms responsible for the association are not established. Stimulated neutrophils are capable of inducing genotoxic effects, such as DNA strand breaks (*32, 72*), sister chromatid exchanges (*73*) and mutation (*74, 75*), and promotion of neoplastic transformation in nearby cells (*74, 76*). MPO-generated reactive chlorinating species have been shown to be mutagenic to bacteria (*77*), and MPO has been indirectly implicated in playing a role in carcinogenesis through both activation of procarcinogens to genotoxic intermediates and the potentiation of xenobiotic carcinogenicity (*78, 79*). Recent genetic studies implicate MPO in the

development of lung cancer. Individuals possessing a relatively abundant polymorphism associated with diminished MPO expression demonstrated a dramatic reduction in the relative risk for development of nonsmall cell lung cancers (*80*). The results of the present study suggest that a potential mechanism accounting for some of these observations may be through MPO-dependent damage of DNA by •OH.

Similarly, although a potential role for eosinophils in promoting DNA oxidative damage, carcinogenesis, or metastatic conversion of a malignancy has not yet been explored, the idea is not without precedent. Multiple chronic parasitic infections are causally linked to subsequent development of cancer and represent a leading cause of cancer mortality in some underdeveloped countries (e.g., *Schistosoma haematobium* and bladder cancer, or *Opisthorcis viverrini* and cholangiocarcinoma) (*21, 81*−*84*). Eosinophilic infiltration is a hallmark of these disorders. Interestingly, recent studies demonstrated that the vast majority of breast cancer biopsies contain eosinophils and intensely stain for EPO (*85*). Significant increases in the content of •OH-generated lesions in the DNA from cancerous and metastatic breast tissues have been noted (*12, 13*). Studies of hormone-responsive tissues such as the breast and uterus suggest that eosinophil migration occurs in response to estrogen administration (*86*), and it has long been recognized that elevated peroxidase activity is present in human breast cancers (*87, 88*). Moreover, eosinophil infiltration in lymphomas and malignancies on cutaneous or mucosal surfaces are common (*29, 30*). The function of eosinophils in cancer is uncertain. Although it is assumed that they play a protective role in tissue surveillance, the results of the present study raise the interesting possibility that they may also potentially contribute to DNA oxidative damage, cancer development, or metastases formation.

Previous studies examining DNA damage by activated neutrophils have primarily focused on their ability to promote DNA strand breaks in neighboring cells (*32, 33, 36, 37*). DNA damage in these studies is typically blocked by catalase and significantly increased by addition of Fe^{2+} salts into the media (*35, 36, 89*). The results of the present study suggest that halide-dependent •OH formation is a free metal ion-independent mechanism that may contribute to DNA damage observed in these models. Consistent with this suggestion, several investigators have observed that repair of DNA strand breaks induced by stimulated neutrophils is slower than the repair rate of similar levels of DNA damage induced by nonphagocytic sources of H_2O_2 (*72, 89*). Furthermore, although HOCl does not directly generate DNA strand breaks in human lymphocytes, activation of neutrophils in the presence of MPO inhibitors is reported to significantly decrease the extent of DNA strand breaks, as well as increase the rate of repair (*72*). Thus, in addition to their potential role in damaging DNA and free nucleobases through •OH formation, peroxidase-generated hypohalous acids might also potentially contribute to increased risk for cancer development by inhibiting DNA repair mechanisms at the sites of oxidant production.

One striking feature of the cytotoxicity studies was the apparent ability of injured cells to undergo repair and ultimately become mitotically active, as assessed by clonogenic survival assays (Table 1). For example, although almost 40% of preloaded [^{14}C]adenine was released from intracellular pools of HA1 cells exposed to both hyperoxia and the HOCl-generating system, less than 20% of these cells ultimately died (i.e., became clonogenically incompetent, Table 1). These results suggest that at sites of chronic inflammation, a "gray zone" will likely exist where DNA, RNA, and free nucleotide damage occurs, but which is subsequently processed and repaired. The chronic nature of many inflammatory conditions associated with enhanced risk for cancer development (e.g., inflammatory bowel diseases, hepatitis, numerous parasitic infections) often spans decades. Thus, even a modest lack of fidelity in repair mechanisms could potentially result in mutations at critical sites which ultimately lead to malignant transformation and cancer development.

One key question was whether •OH generated by interaction of $O_2^{\bullet-}$ and hypohalous acid could damage cellular DNA. The results of the present study demonstrate that DNA, RNA, and free cytosolic nucleotides are all potential targets for •OH-mediated damage in cells exposed to an exogenous hypohalous acid-generating system, particularly under conditions of enhanced intracellular $O_2^{\bullet-}$ production (Table 1). Mitochondrial DNA, and to a lesser extent RNA and cytosolic nucleobases, demonstrated the greatest levels of 8-OHG following exposure of cells to the MPO−H_2O_2−Cl^- system. A likely explanation for the enhanced susceptibility of mitochondrial DNA to oxidative damage is that mitochondria serve as a primary source of intracellular $O_2^{\bullet-}$ production. This would facilitate •OH formation in close proximity to mitochondrial DNA. In addition, mitochondrial DNA does not bear a protective coat of highly basic histone proteins; thus, protection from reactive oxidants such as hypohalous acids is far less than that observed in the nucleus. Finally, DNA repair mechanisms in mitochondria are less efficient than those in the nucleus (*68*).

It is also interesting that 8-OHG was formed in the cytosolic pool of cells exposed to the peroxidase−H_2O_2−halide system of leukocytes (Table 1). Oxidized nucleobases from cellular pools may be incorporated into DNA, leading to substitution mutations in the case of 8-OHG (*14, 15*). Although there are DNA repair systems which recognize 8-OHG with high affinity, the fidelity of these repair enzymes is not absolute, and increased cancer risk with accompanying increased levels of oxidative DNA damage is known to occur when defects in the DNA repair systems are present (*90, 91*).

Few studies have reported determination of 8-OHG in RNA. The highly reactive nature of •OH, and the subsequent limited diffusional distance of the species before productive encounter, suggests that much of the •OH generated in cells exposed to HOCl during hyperoxia also occurred in the cytosol. Redox cycling of cytosolic flavoproteins may serve as a source of $O_2^{\bullet-}$ under these conditions (*69*). Because of the very rapid removal of 8-OHG from DNA, but not RNA (*92*), quantification of 8-OHG in RNA might represent a useful complimentary method for evaluating the intracellular source of cellular oxidative damage induced in cells. Whether oxidative modification of RNA plays a role in transcriptional regulation at sites of inflammation remains to be determined.

In summary, the present study identifies a novel mechanism for 8-OHG formation by activated phagocytic cells. The role of peroxidase-generated oxidants in contributing to the etiology of inflammation-related cancers is unknown.

We focused on the formation of 8-OHG as a marker of •OH formation and DNA damage because this mutagenic base both is implicated in cancer development and is easily quantified by sensitive and specific methods. Although 8-OHG represents one of the more abundant •OH-mediated products formed during DNA oxidation, numerous additional types of base modifications occur as a result of DNA exposure to •OH. Indeed, we observed multiple additional peaks of unknown identity during HPLC-EC analyses, and even more electrochemically silent oxidation products of DNA are undoubtedly formed. It is tempting to speculate that hypohalous acids might act directly on DNA, RNA, or cytosolic nucleobases, generating halogenated oxidation products. Future studies in this area are warranted since these might serve as powerful tools to identify a direct role for MPO and EPO in promoting oxidative damage of nucleotides in vivo.

ACKNOWLEDGMENT

Mass spectrometry experiments were performed at the Cleveland Clinic Foundation Mass Spectrometry Core Facility.

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BI992809Y

Biochemistry **2001,** *40,* 2041−2051

Eosinophil Peroxidase Catalyzes Bromination of Free Nucleosides and Double-Stranded DNA†

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Received August 17, 2000; Revised Manuscript Received November 7, 2000

ABSTRACT: Chronic parasitic infections are a major risk factor for cancer development in many underdeveloped countries. Oxidative damage of DNA may provide a mechanism linking these processes. Eosinophil recruitment is a hallmark of parasitic infections and many forms of cancer, and eosinophil peroxidase (EPO), a secreted hemoprotein, plays a central role in oxidant production by these cells. However, mechanisms through which EPO may facilitate DNA oxidation have not been fully characterized. Here, we show that EPO effectively uses plasma levels of bromide as a cosubstrate to brominate bases in nucleotides and double-stranded DNA, forming several stable novel brominated adducts. Products were characterized by HPLC with on-line UV spectroscopy and electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS). Ring assignments for brominated purine bases as their 8-bromo adducts were identified by NMR spectroscopy. Using stable isotope dilution LC/ESI/MS/MS, we show that while guanine is the preferred purine targeted for bromination as a free nucleobase, 8-bromoadenine is the major purine oxidation product generated following exposure of double-stranded DNA to either HOBr or the EPO/H_2O_2/Br^- system. Bromination of nucleobases was inhibited by scavengers of hypohalous acids such as the thioether methionine, but not by a large molar excess of primary amines. Subsequently, *N*-monobromoamines were demonstrated to be effective brominating agents for both free nucleobases and adenine within intact DNA. A rationale for selective modification of adenine, but not guanine, in double-stranded DNA based upon stereochemical criteria is presented. Collectively, these results suggest that specific brominated DNA bases may serve as novel markers for monitoring oxidative damage of DNA and the nucleotide pool by brominating oxidants.

There is a well-established link between chronic inflammation and the development of certain forms of cancer (*1*−*7*). For example, multiple chronic parasitic infections are causally linked to cancer development (e.g., *Schistosoma haematobium* and bladder cancer and *Opisthorcis viverrini* and cholangiocarcinoma) and represent a leading cause of cancer mortality in some underdeveloped countries (*7*−*10*). Eosinophilic infiltration is a hallmark of these disorders (*11*). Similarly, chronic hepatitis and forms of inflammatory bowel disease (ulcerative colitis) are associated with dramatic increases in the risk for development of specific cancers [i.e., hepatocellular carcinoma and adenocarcinoma of the colon, respectively (*11*)]. The mechanisms linking the associations between chronic inflammation and cancer development are unknown. However, the prodigious capacity of leukocytes to generate free radicals and reactive oxidant species (*12*−*15*), coupled with the significant data linking DNA oxidative damage to mutagenesis and cancer development (*16*−*20*), strongly suggests that leukocyte-dependent oxidative damage of DNA may be a mechanism.

During leukocyte activation at sites of inflammation, components of the NADPH oxidase complex become phosphorylated and assemble at the plasma membrane. The activated complex subsequently catalyzes the rapid reduction of molecular oxygen into superoxide ($O_2^{\bullet-}$) during a respiratory burst (*21*). Reduced oxygen species formed by leukocytes, such as $O_2^{\bullet-}$ and its dismutation product, hydrogen peroxide (H_2O_2), may then be converted into more reactive oxidizing species with potential for promoting DNA oxidation during chronic inflammation. In support of this hypothesis, in vitro studies demonstrate that stimulated leukocytes are capable of inducing genotoxic effects, such as DNA strand breaks (*22*, *23*), sister chromatid exchanges (*24*), mutation (*25*−*27*), and promotion of neoplastic transformation in nearby cells (*25*, *28*). Halogenating oxidants have

† This work was supported by a grant from the Department of Defense (BC980289) and by grants from the National Institutes of Health (HL62526 and HL61878 to S.L.H. and HL58758 to J.Q.). Z.S. is a recipient of a Medical Research Fellowship from the Jane Coffin Childs Memorial Fund for Medical Research.

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10.1021/bi001961t CCC: $20.00
Published on Web 01/26/2001

also been shown to be mutagenic to bacteria (*29*, *30*). A role in cancer development has also been suggested for the leukocyte peroxidase myeloperoxidase (MPO)[1] based upon its ability to convert procarcinogens into genotoxic intermediates and the modification of certain xenobiotics into more potent carcinogens (*31*−*34*). Recent studies also suggest a genetic link between MPO and the risk for developing cancer. The incidence of a specific polymorphism in the promotor region of the MPO gene has been demonstrated to be in genetic disequilibrium with the incidence of several cancers (*35*, *36*).

The precise chemical mechanisms linking inflammation, leukocyte activation, and DNA oxidative damage in vivo have not yet been fully characterized. Most studies have focused on the ability of free transition metal ions, like Fe^{2+} or Cu^{2+}, to catalyze conversion of leukocyte-generated reduced oxygen species (i.e., $O_2^{\bullet-}$ and H_2O_2) into the more reactive hydroxyl radical ($^{\bullet}OH$) through classic Haber-Weiss and Fenton chemistries (eq 1) (*37*−*39*).

$$O_2^{\bullet-} + H_2O_2 \xrightarrow{M^{2+}} {}^{\bullet}OH + OH^- + O_2 \qquad (1)$$

Eosinophils and neutrophils are endowed with distinct peroxidases, MPO and eosinophil peroxidase (EPO), respectively, which can utilize halides (X^-) as substrates to form hypohalous acids (HOX) (eq 2) (*13*, *40*).

$$X^- + H_2O_2 + H^+ \rightarrow HOX + H_2O \ (X = Cl \text{ or } Br) \qquad (2)$$

These potent cytotoxic oxidants are microbicidal and are presumed to play a key role in the normal functions of leukocytes during innate host defenses (*41*−*43*). Recently, activated eosinophils and neutrophils (as well as their corresponding isolated peroxidases, EPO and MPO, respectively) were shown to promote oxidative damage of DNA, RNA, and the nucleotide pool of target cells through halide-dependent formation of $^{\bullet}OH$ (eq 3) (*44*).

$$O_2^{\bullet-} + HOX \rightarrow {}^{\bullet}OH + X^- + O_2 \ (X = Cl \text{ or } Br) \qquad (3)$$

This reaction is analogous to the Haber-Weiss reaction (eq 1) where H_2O_2 is replaced with HOCl or HOBr. In the absence of metal ions, eq 3 is at least 6 orders of magnitude faster than the Haber-Weiss reaction (*45*−*47*).

Although the contribution of oxidative processes to carcinogenesis is now widely accepted, the precise chemical pathways involved remain unclear. A powerful method for unraveling the mechanisms through which DNA damage occurs is through the detection of distinct stable markers of free radical reactions. For example, markers of DNA damage by $^{\bullet}OH$, reactive nitrogen species, reactive chlorinating species, and aldehydes have been used to implicate specific chemical mechanisms of DNA damage in vivo (*48*−*55*). Although eosinophil recruitment is characteristic of many cancers (*56*−*58*) and numerous chronic parasitic infections are associated with an increased risk for development of cancer (*7*−*10*, *59*), no stable DNA oxidation products specific for eosinophils have been described.

Nearly two decades ago, the unique ability of eosinophils to generate brominating oxidants via the EPO/H_2O_2/Br^- system was first reported (*60*). Recently, the sensitivity and specificity of mass spectrometry has permitted the direct demonstration that brominating oxidants are a distinct class of oxidants formed by eosinophils in vivo (*61*). Since EPO is the only mammalian enzyme known to selectively generate reactive brominating species (*60*) at physiological concentrations of halides (100 mM Cl^-, 20−150 μM Br^-, and 0.1−0.6 μM I^- in plasma; *61*, and references therein), brominated products have the potential to serve as molecular markers that identify sites of EPO-mediated oxidative damage. In this study, we examined the ability of brominating oxidants and isolated EPO to promote oxidation of nucleosides and double-stranded DNA. Multiple distinct brominated products of nucleosides and DNA were observed using a combination of various mass spectrometric and NMR methods. The chemical reactions that are involved are characterized, and preferred targets for modification in double-stranded DNA are defined. A rationale for selective modification of adenine, but not guanine, in double-stranded DNA is also presented, based upon stereochemical criteria. The specific brominated nucleoside adducts that are identified may thus serve as molecular markers that permit identification of sites where brominating oxidants contribute to DNA damage.

EXPERIMENTAL PROCEDURES

Materials

Organic solvents (HPLC grade), H_2O_2 (30%; ACS grade), H_3PO_4, NaH_2PO_4, and Na_2HPO_4 were obtained from Fisher Chemical Co. (Pittsburgh, PA). Chelex-100 resin (200−400 mesh, sodium form) was obtained from Bio-Rad (Hercules, CA). Methanesulfonic acid and bromine were purchased from Fluka Chemical Co. (Ronkonkoma, NY). Heavy isotope-labeled adenosine (U-$^{13}C_{10}$, 98%; U-$^{15}N_5$, 98%) was obtained from Cambridge Isotope Laboratories (Andover, MA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

Methods

General Procedures. Porcine EPO was isolated using the method of Jorg (*62*). Peroxidase activity during purification was monitored by the guaiacol oxidation assay (*41*). Purity of EPO preparations was assured before use by demonstrating an RZ of >0.95 (A_{415}/A_{280}), SDS−PAGE analysis with Coomassie Blue staining, and in-gel tetramethylbenzidine peroxidase staining to confirm the absence of contaminating MPO activity (*63*). MPO was initially purified from detergent extracts of human leukocytes by sequential lectin affinity and gel filtration chromatography as described previously (*64*). Trace levels of contaminating EPO were then removed by cation exchange chromatography (*65*). The purity of isolated MPO was established by demonstrating an RZ of >0.85 (A_{430}/A_{280}), SDS−PAGE analysis with Coomassie Blue staining, and in-gel tetramethylbenzidine peroxidase staining to confirm the absence of contaminating EPO activity (*63*). Enzyme concentrations were determined spec-

[1] Abbreviations: 8-BrA, 8-bromoadenine; 8-BrdA, 8-bromo-2′-deoxyadenosine; Br-dC, bromo-2′-deoxycytidine; 8-BrG, 8-bromoguanine; 8-BrdG, 8-bromo-2′-deoxyguanosine; Br-dT, bromo-2′-deoxythymidine; EPO, eosinophil peroxidase; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; MS, mass spectrometry; *m/z*, mass-to-charge ratio; $[M + H]^+$, molecular ion; MPO, myeloperoxidase; MRM, multiple-reaction monitoring; SIM, selected ion monitoring; TFA, trifluoroacetic acid; UV, ultraviolet−visible.

trophotometrically utilizing extinction coefficients of 89 000 and 112 000 M^{-1} cm^{-1}/heme for MPO (*66*) and EPO (*67*, *68*), respectively. The concentration of the MPO dimer was calculated as half the indicated concentration of the heme-like chromophore.

Buffers were prepared with Chelex-treated distilled deionized water. HOBr, free of Br^- and bromate, was prepared the day of use from liquid bromine as described previously (*69*). HOBr was quantified spectrophotometrically (ϵ_{331} = 315 M^{-1} cm^{-1}) as its conjugate base, OBr^- (*70*). Fresh stock solutions of *N*-bromotaurine or *N,N*-dibromotaurine were prepared in phosphate buffer (20 mM, pH 7.0) by incubating the HOBr/OBr^- mixture with either a 100-fold molar excess of taurine or 0.5 molar equiv of taurine, respectively, immediately prior to use. The concentrations of H_2O_2 (ϵ_{240} = 39.4 M^{-1} cm^{-1}) (*71*), *N*-bromoamine (ϵ_{288} = 430 M^{-1} cm^{-1}) (*72*), and *N,N*-dibromoamine (ϵ_{336} = 371 M^{-1} cm^{-1}) (*72*) were determined spectrophotometrically. Production of H_2O_2 by the glucose/glucose oxidase system was assessed by oxidation of Fe(II) and formation of an Fe(III)−thiocyanate complex (*73*). ^{13}C- and ^{15}N-labeled bromoadenine (Br[$^{13}C_5$,$^{15}N_5$]A) was synthesized for use as an internal standard by the reaction of ^{13}C- and ^{15}N-labeled adenosine with an equimolar amount of HOBr in 50 mM phosphoric acid, and the products were identified and quantified by HPLC with on-line UV (254 nm) spectroscopy and electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS).

Bromination of Free Deoxyribonucleosides and Calf Thymus DNA. The DNA bases 2′-deoxyadenosine, 2′-deoxyguanosine, 2′-deoxycytidine, and 2′-deoxythymidine (dA, dC, dG, and dT, respectively) (10 mM) or Chelex-treated calf thymus DNA (1 mg/mL) was brominated by addition of oxidant (HOBr, *N*-monobromoamine, *N,N*-dibromoamine, H_2O_2, or glucose to the glucose/glucose oxidase system) in sodium phosphate buffer (20 mM, pH 7.0, unless otherwise indicated) at 37 °C for either 60 min or overnight under the conditions described in the figure legends. Reactions were stopped by addition of methionine (10 mM). Experiments utilizing the glucose/glucose oxidase system for H_2O_2 generation were performed in the presence of 100 μg/mL glucose and either 20 (0.005 unit/mL) or 100 ng/mL (0.025 unit/mL) glucose oxidase (grade II, Boehringer Mannheim, Indianapolis, IN) at 37 °C overnight. Preliminary studies demonstrated that under these conditions, constant fluxes of H_2O_2 at approximately 10 and 50 μM/h, respectively, are formed.

Sample Preparation. Following oxidation, DNA was pelleted at 0 °C by adding 100% ethanol and then washed with 70% ethanol. Samples were then resuspended in 500 μL of H_2O, and the synthetic ring-labeled internal standard (Br[$^{13}C_5$,$^{15}N_5$]A) was added. DNA was then hydrolyzed with methanesulfonic acid (100 μM) at 90 °C for 2 h in gastight vials evacuated of air and under a blanket of argon. Hydrolysates were resuspended in 500 μL of Chelex-treated H_2O. The adenine content was then quantified by reverse phase HPLC with UV detection (254 nm). Prior to LC/ESI/MS/MS analysis, natural and heavy isotope-labeled 8-bromoadenine (8-BrA) and 8-bromoguanine (8-BrG) were partially purified from the DNA hydrolysates by passage over a Supelclean LC-C_{18} SPE minicolumn (Supelclean LC-18 SPE tubes, 3 mL; Supelco Inc., Bellefonte, PA). Briefly, the hydrolysates were loaded onto minicolumns which had been pre-equilibrated with 0.1% trifluoroacetic acid (TFA). Salts and the majority of DNA bases were eluted from the column through sequential washes (2 mL) of 0.1% TFA. Brominated bases were then eluted with 2 mL of 20% methanol in 0.1% (v/v) trifluoroacetic acid (TFA in H_2O). Fractions were dried, reconstituted with H_2O, and subjected to LC/ESI/MS/MS analysis.

Reverse Phase HPLC Analysis of Modified DNA Bases. Analysis of free deoxyribonucleosides was performed on a Beckman Gold HPLC system equipped with a photodiode array detector. Separations were performed on a C18 column (Beckman Ultrasphere, 5 μm, 4.6 mm × 250 mm) equilibrated with solvent A [0.1% TFA (pH 2.5)]. The products were eluted at a flow rate of 1 mL/min with a linear gradient generated with solvent B [0.1% TFA in methanol (pH 2.5)] as follows: 0% solvent B for 5 min, from 0 to 100% solvent B over the course of 30 min, and 100% solvent B for 10 min. Products were monitored on a diode array detector and quantified at 254 nm employing standard curves constructed with authentic synthetic standards.

Mass Spectrometry. Mass spectrometric analyses were performed on a Quattro II triple-quadrupole mass spectrometer (Micromass, Inc., Altrincham, U.K.) equipped with an electrospray ionization (ESI) probe and interfaced with an HP 1100 HPLC system (Hewlett-Packard, Wilmington, DE) with a photodiode array detector. DNA bases were resolved on an Ultrasphere C18 column (Beckman, 5 μm, 4.6 mm × 250 mm) at a flow rate of 1 mL/min and a linear gradient between H_2O (with 0.3% formic acid) and methanol (with 0.3% formic acid) over the course of 30 min. The column eluent was split (970 μL/min to the UV detector and 30 μL/min to the mass detector) and analyzed by the mass spectrometer in the positive ion mode with a cone potential of 50 eV. Analytes were detected using the total ion scan mode or the selected ion monitoring (SIM) mode, or with multiple-reaction monitoring (MRM) as indicated in the text and legends.

Quantification of 8-BrA was performed following resolution on a Luna C18 column (Phenomenex, 5 μm, 1.0 mm × 30 mm) at a flow rate of 25 μL/min and an isocratic condition of 30% CH_3CN in H_2O with 0.05% formic acid and 0.005% TFA. 8-BrA and its corresponding heavy isotope-labeled internal standard, 8-Br[$^{13}C_5$,$^{15}N_5$]A, were detected using positive ion electrospray ionization mass spectrometry in the MRM mode. The transitions that were monitored were those between the molecular cation of the ^{79}Br isotopomer and the characteristic brominated daughter ion formed from loss of NH_3 [i.e., mass-to-charge ratio (*m/z*) 214 → 197 for BrA and 224 → 206 for 8-Br[$^{13}C_5$,$^{15}N_5$]A].

NMR Studies. NMR samples were prepared by dissolving deoxyribonucleic acids and HPLC-purified brominated adducts in 100% D_2O. Analyses were performed at 25 °C with a Varian Inova 500 MHz NMR spectrometer (499.843 MHz for ^1H) equipped with a triple-resonance probe head and a shielded *Z*-gradient unit. ^1H chemical shifts were referenced to external sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 in D_2O. For one-dimensional NMR and two-dimensional NOESY (mixing time of 400 ms) experiments, the intense HOD signal was attenuated by transmitter preirradiation.

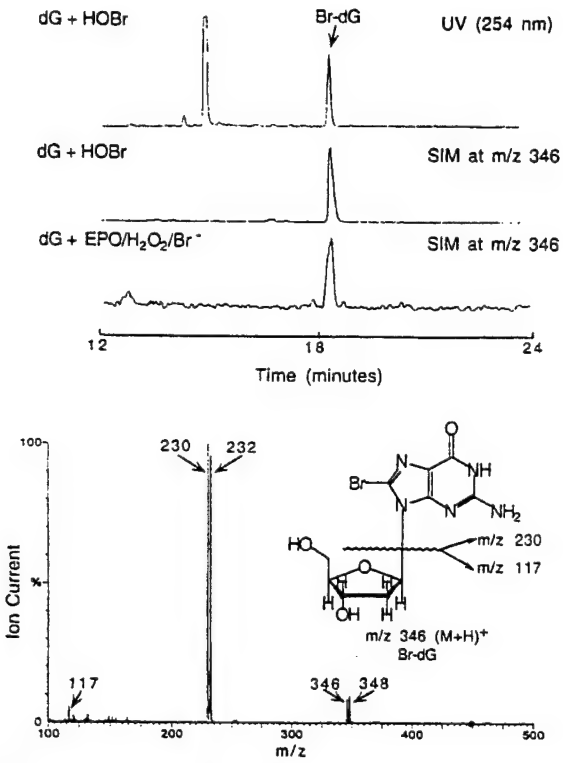

FIGURE 1: Analysis of reaction products formed upon exposure of dG to either HOBr or the EPO/H_2O_2/Br^- system. dG (10 mM) was incubated with either HOBr (5 mM) or the EPO/H_2O_2/Br^- system (57 nM EPO, 100 μM H_2O_2, and 100 μM NaBr) for 1 h at 37 °C in sodium phosphate buffer (20 mM, pH 7.0) supplemented with NaCl (100 mM). Products were subsequently analyzed by reverse phase HPLC with an on-line diode array detector (254 nm trace shown) and positive ion ESI-MS analysis using the single-ion monitoring (SIM) mode at *m/z* 346, the anticipated *m/z* of a protonated monobrominated adduct of dG. The full scan positive ion ESI mass spectrum of the major product generated by EPO (Br-dG, structure and fragmentation pattern shown in the inset) is also shown.

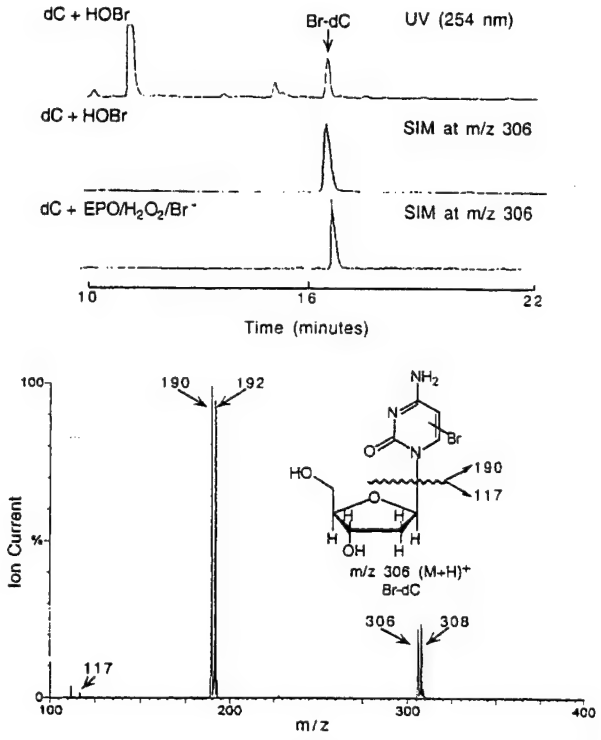

FIGURE 2: Analysis of reaction products formed upon exposure of dC to either HOBr or the EPO/H_2O_2/Br^- system. dC (10 mM) was incubated with either HOBr (5 mM) or the EPO/H_2O_2/Br^- system (57 nM EPO, 100 μM H_2O_2, and 100 μM NaBr) for 1 h at 37 °C in sodium phosphate buffer (20 mM, pH 7.0) supplemented with NaCl (100 mM). Products were subsequently analyzed by reverse phase HPLC with an on-line diode array detector (254 nm trace shown) and positive ion ESI-MS analysis using the single-ion monitoring (SIM) mode at *m/z* 306, the anticipated *m/z* of a protonated monobrominated adduct of dC. The full scan positive ion ESI mass spectrum of the major product generated by EPO (Br-dC, structure and fragmentation pattern shown in the inset) is also shown.

RESULTS

Free Nucleosides Are Targets for Oxidation by Eosinophil Peroxidase at Plasma Levels of Halides. In initial experiments, each of the individual 2′-deoxynucleosides (dG, dC, dA, or dT) was incubated with isolated EPO, H_2O_2, and plasma levels of Br^- (100 μM) and Cl^- (100 mM), and then the reaction products were analyzed by reverse phase HPLC with on-line UV detection (254 nm) and electrospray ionization mass spectrometry (ESI/MS) in the positive ion mode (Figures 1−4). Analysis of the oxidation products formed by exposure of dG to either HOBr or the EPO/H_2O_2/Br^- system in phosphate buffer at neutral pH demonstrated the formation of a major new product with a distinct retention time (Figure 1). The positive ion mass spectrum of the oxidized base generated by the EPO system is shown in the bottom panel of Figure 1 and is consistent with a monobrominated derivative of dG possessing a molecular ion $[M + H]^+$ with a mass-to-charge ratio (*m/z*) of 346. The mass spectrum also demonstrates the isotopic cluster expected for a monobrominated compound (1:1, M:M + 2), with ions of near equal relative abundance at *m/z* 346 ($[M + H]^+$ of the ^{79}Br-containing isotopomer) and *m/z* 348 ($[M + H]^+$ of the ^{81}Br-containing isotopomer). The mass spectrum also revealed fragment ions consistent with cleavage of the sugar−purine bond, generating the anticipated daughter ions arising from the 2′-deoxyribose group (*m/z* 117) and a monobrominated guanine moiety (*m/z* 230) (Figure 1, bottom panel and inset). Finally, analysis of the oxidation products formed by either reagent HOBr or the EPO/H_2O_2/Br^- system using LC/ESI/MS with monitoring in the selected ion monitoring mode (SIM) at *m/z* 346 $[M + H]^+$ demonstrated a single major product with an identical retention time (Figure 1). These results demonstrate that a stable ring-brominated adduct was formed following exposure of free dG to EPO-generated HOBr (Figure 1).

Similar results were obtained when dC was incubated with either reagent HOBr or isolated EPO in the presence of H_2O_2 and plasma levels of halides (Figure 2). A single major new product with a distinct retention time was observed during analysis by reverse phase HPLC with on-line UV detection (254 nm) and LC/ESI/MS analysis monitoring at *m/z* 306, the anticipated $[M + H]^+$ for the monobrominated species (Figure 2). Again, the full-scale positive ion ESI/MS spectrum of the major oxidation product was consistent with the formation of a monobrominated base, as illustrated in Figure 2.

When dA was incubated with HOBr in phosphate buffer at pH 7.0, no detectable products were apparent during HPLC

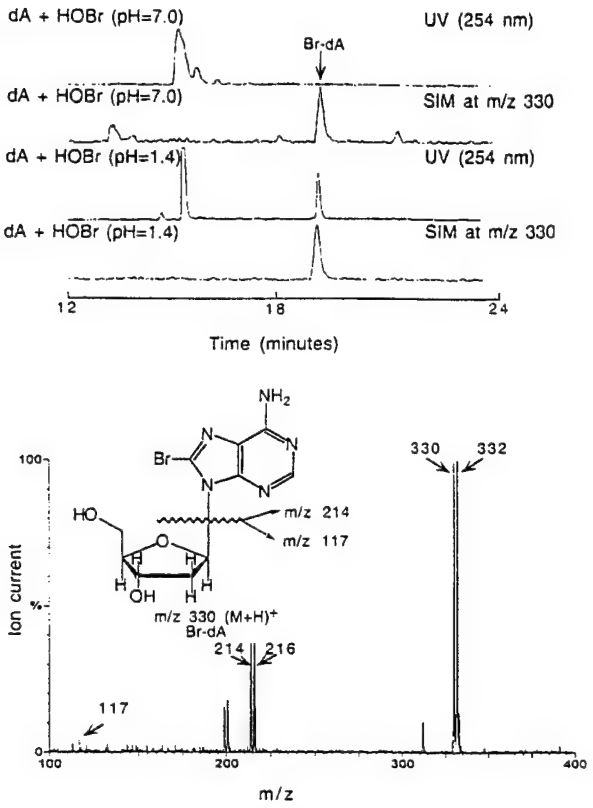

FIGURE 3: Analysis of reaction products formed upon exposure of dA to either HOBr or the EPO/H_2O_2/Br^- system. dA (10 mM) was incubated with HOBr (5 mM) for 1 h at 37 °C in sodium phosphate buffer (20 mM, pH 7.0 or 1.4) supplemented with NaCl (100 mM). In parallel, dA (10 mM) was incubated with the EPO/H_2O_2/Br^- system (57 nM EPO, 100 μM H_2O_2, and 100 μM NaBr) for 1 h at 37 °C in sodium phosphate buffer (20 mM, pH 7.0) supplemented with NaCl (100 mM). Products were subsequently analyzed by reverse phase HPLC with an on-line diode array detector (254 nm trace shown) and positive ion ESI-MS analysis using the single-ion monitoring (SIM) mode at *m/z* 330, the anticipated *m/z* of a protonated monobrominated adduct of dA, as described in Experimental Procedures. The full scan positive ion ESI mass spectrum of the major product generated by EPO (Br-dA, structure and fragmentation pattern shown in the inset) is also shown.

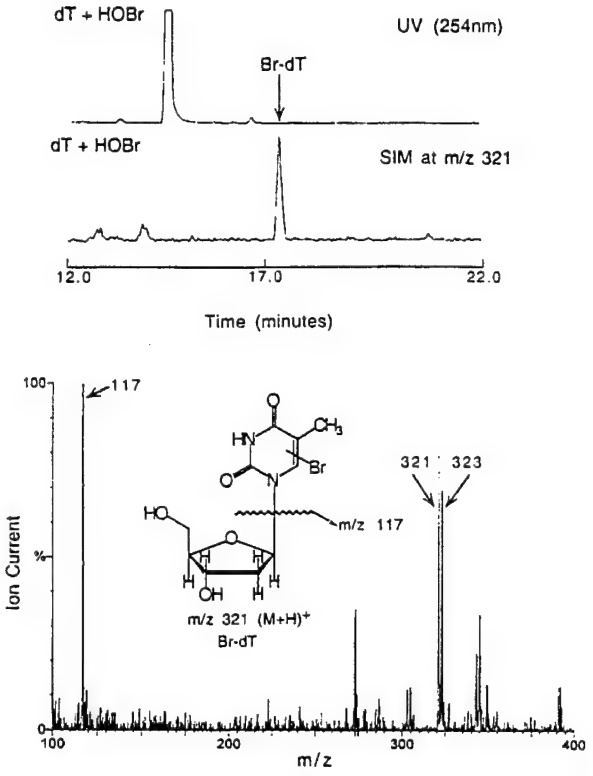

FIGURE 4: Analysis of reaction products formed upon exposure of dT to either HOBr or the EPO/H_2O_2/Br^- system. dT (10 mM) was incubated with either HOBr (5 mM) or the EPO/H_2O_2/Br^- system (57 nM EPO, 100 μM H_2O_2, and 100 μM NaBr) for 1 h at 37 °C in sodium phosphate buffer (20 mM, pH 7.0) supplemented with NaCl (100 mM). Products were subsequently analyzed by reverse phase HPLC with an on-line diode array detector (254 nm trace shown) and positive ion ESI-MS analysis using the single-ion monitoring (SIM) mode at *m/z* 321, the anticipated *m/z* of a protonated monobrominated adduct of dT. The full scan positive ion ESI mass spectrum of the major product generated by EPO (Br-dT, structure and fragmentation pattern shown in the inset) is also shown.

analysis by UV using a photodiode array detector (data for 254 nm shown); however, a product with the appropriate *m/z* of a monobrominated dA derivative was readily detected by LC/ESI/MS during SIM at *m/z* 330, the anticipated *m/z* for a monobrominated adduct. When dA was exposed to HOBr under acidic conditions, significant levels of Br-dA were apparent during HPLC analysis by both UV and ESI/MS detection (Figure 3). The molecular ion and fragmentation pattern of the product was again consistent with formation of the monobrominated species (Figure 3, bottom panel). Finally, exposure of dT to the major oxidant of the EPO/H_2O_2/Br^- system, HOBr, at neutral pH produced low levels of monobrominated dT (Br-dT) which was only detected by LC/ESI/MS in SIM at *m/z* 321 (anticipated *m/z* for the monobrominated adduct) (Figure 4). At low pH, Br-dT was formed in high yield, along with numerous other mono- and dibrominated degradation products. Collectively, these results demonstrate that all four free deoxyribonucleosides can serve as targets for bromination. However, the yields of Br-dG and Br-dC were substantially higher at neutral pH than those of Br-dA and Br-dT, suggesting that they will serve as primary targets among the free nucleobases.

Structural Identification of Brominated Purine Bases. Preliminary studies suggested that the monobrominated pyrimidine products that are formed are acid labile. Since our long-term interest is to identify stable brominated nucleotide adducts which might serve as in vivo markers for the detection of DNA damage by EPO-generated brominating oxidants, we focused our subsequent studies on structurally characterizing the monosubstituted brominated adducts of purines formed following exposure of bases to EPO-generated HOBr. To identify the sites on the heterocyclic rings where bromine was covalently attached, we initially utilized ^1H NMR methods. The one-dimensional spectra of Br-dG and the parent base, dG, are shown in Figure 5. Inspection of the spectra reveals that the H_8 proton is lost in the brominated adduct, confirming the ring assignment of the monobrominated species as 8-Br-2′-dG (also known as Br-dG). Analysis of the proton NMR spectra (one-dimensional) of monobrominated and native dA did not permit unambiguous assignment of the location for ring bromination. Subsequent two-dimensional NOESY analyses

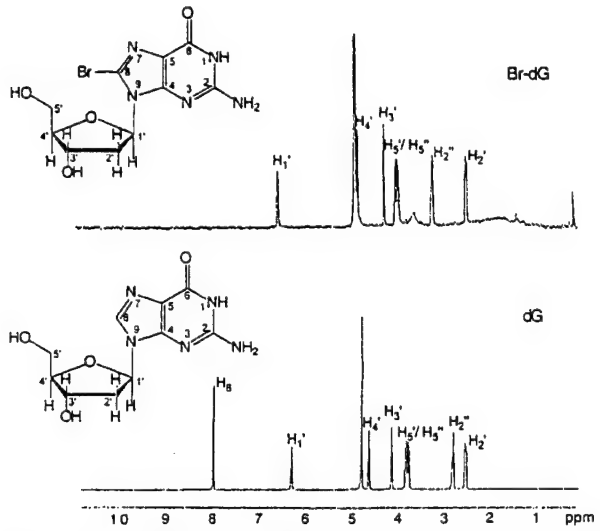

FIGURE 5: ^{1}H NMR spectra of BrdG and dG. The monobrominated oxidation product of dG formed following exposure to the EPO/H_2O_2/Br^- system (*m/z* 346) was isolated by reverse phase HPLC, dried, and then analyzed by ^{1}H NMR in D_2O as described in Experimental Procedures. For comparison, the ^{1}H NMR spectrum of dG is shown. Note the disappearance of the resonance derived from the H_8 proton in the ^{1}H NMR spectrum of BrdG, compared to that of dG.

demonstrated that the aromatic H_8 proton of dA exhibits several NOE cross-peaks to the adjacent sugar ring protons (Figure 6, top panel), while these NOE connections disappear in Br-dA (Figure 6, bottom panel). Thus, the H_8 position of dA is replaced with bromine, and the monobrominated dA species that forms is 8-Br-2′-dA (also known as Br-dA).

Characterization of Reaction Requirements and Reactive Intermediates Involved in Br-dG Formation by EPO. We next examined the reaction requirements for Br-dG formation by isolated EPO. Following incubation of dG with EPO, a H_2O_2-generating system (glucose and glucose oxidase; ~10 μM H_2O_2/h under the conditions that were employed), and plasma levels of halides, Br-dG was formed (Figure 7, top panel, complete system). The time course for EPO-dependent bromination of dG under these conditions is illustrated in Figure 8. Near-maximal Br-dG formation was observed within 2 h. If 1 mol of H_2O_2 is required to generate 1 mol of brominating equivalent from EPO, and 1 mol of brominating equivalent is used to generate Br-dG, then the overall yield of EPO-dependent bromination of dG was ~6.5% under the conditions employed here. Formation of Br-dG required the presence of each component of the EPO/H_2O_2/Br^- system. Moreover, addition of either heme inhibitors, like azide, or scavengers of reactive halogenating species, like the thioether methionine, completely ablated formation of the brominated base (Figure 7, top panel). In contrast, BrdG formation still was observed in reaction mixtures containing N_α-acetyllysine, a low-molecular weight surrogate for protein amino groups. These results suggested that *N*-bromoamines might promote nucleotide bromination reactions. Exposure of excess dG to an equivalent amount of distinct brominating intermediates, including HOBr and OBr^-, *N*-monobromotaurine, *N*,*N*-dibromotaurine, and HOBr with Br^- (a Br_2-containing system), revealed that *N*-bromoamines were the preferred brominating intermediates (~40% overall yield, per mole of brominating equivalent)

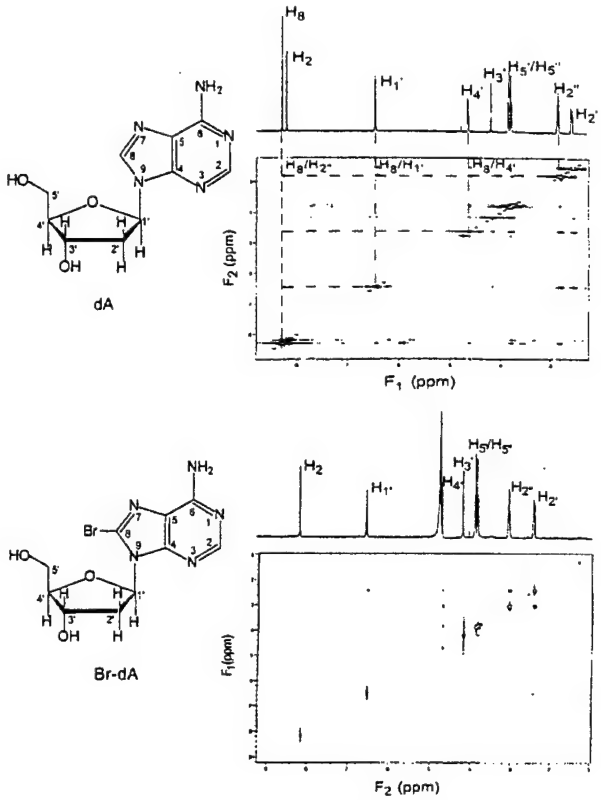

FIGURE 6: Two-dimensional NOESY spectra of dA and Br-dA. The monobrominated oxidation product of dA formed following exposure to the EPO/H_2O_2/Br^- system (*m/z* 330) was isolated by reverse phase HPLC. Both dA and the monobrominated product were each then analyzed by two-dimensional NMR as described in the text. Note that the two-dimensional NOESY spectrum of dA shows the NOE cross-peak between H_8 and $H_{2'}$ which are spatially close. In contrast, the two-dimensional NOESY spectrum of Br-dA shows the disappearance of H_8 and its NOE connection with $H_{2'}$.

(Figure 7, bottom panel). Finally, the bromide concentration dependence of Br-dG formation by the EPO/H_2O_2 system in medium containing 100 mM Cl^- was examined and is illustrated in Figure 9. Even in the face of a >1000-fold molar excess of Cl^-, EPO effectively generates Br-dG across the physiological concentration range of Br^- (20−150 μM).

8-Bromoadenine Is the Major Stable Purine Oxidation Product Formed following Exposure of DNA to the HOBr/OBr^- System. A critical question was whether bases in double-stranded DNA would serve as a target for bromination. To test this, we exposed calf thymus DNA to low levels of reagent HOBr and then DNA was recovered, washed, hydrolyzed, and analyzed by LC/ESI/MS. Interestingly, BrA, but not BrG, was detected in the SIM mode while monitoring at *m/z* 214 and 230, the *m/z* anticipated for the molecular ions of BrA and BrG, respectively (Figure 10). The identity of the ion at *m/z* 214 as BrA was confirmed by demonstration of the retention time being identical to that of authentic BrA (not shown) and analysis of the full scan positive ion mass spectrum of the analyte, which revealed the characteristic isotopic cluster of the monobrominated adduct (Figure 10, inset).

Demonstration That the EPO/H_2O_2/Br^- System Generates BrA in Double-Helix DNA. To develop a more sensitive and specific method for the identification of BrA in DNA, as

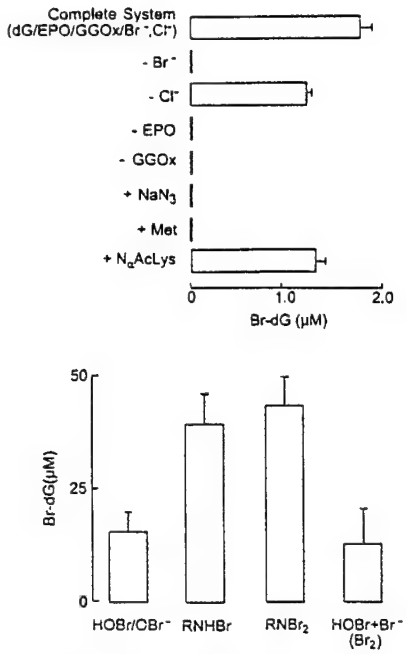

FIGURE 7: Reaction characteristics of 8-BrdG formation by eosinophil peroxidase. (Top) dG (2 mM) was incubated with eosinophil peroxidase (57 nM) and a H_2O_2-generating system (10 μM/h; 100 μg/mL glucose and 20 ng/mL glucose oxidase) in sodium phosphate buffer (20 mM, pH 7.0) supplemented with plasma levels of halides (100 mM NaCl and 100 μM NaBr) at 37 °C (Complete System). Where indicated, components of the Complete System were omitted, or the indicated additions were included in the reaction mixtures: sodium azide (1 mM), methionine (10 mM), or N_α-acetyllysine (1 mM). Reaction products were then analyzed by reverse phase HPLC at 254 nm, and the content of BrdG that formed was determined as described in Experimental Procedures. Data represent the means ± standard deviation (SD) of three independent experiments. (Bottom) dG (2 mM) was incubated with 10 μM (final concentration) $HOBr/OBr^-$, *N*-bromotaurine (RNHBr), *N,N*-dibromotaurine ($RNBR_2$), or $HOBr/OBr^-$ with 100 mM NaBr in reaction buffer [20 mM sodium phosphate (pH 7.0)] at 37 °C for 1 h. Reactions were quenched by addition of methionine (10 mM), and the production of BrdG was assessed by reverse phase HPLC at 254 nm as described in Experimental Procedures. Data represent the means ± SD of three independent experiments.

might be required for detection of the modified base in vivo, we sought to develop a tandem mass spectrometry (MS/MS)-based method for its detection. MS/MS analysis of each isotopomer of BrA produced by EPO-generated HOBr was performed, and the spectra and proposed fragmentation patterns are illustrated in Figure 11. The proposed fragmentation pattern for the monosubstituted adenine is similar to that reported for authentic adenine (*74*). The parent ion (*m/z* 214/216), by the loss of NH_3, forms a major characteristic brominated daughter ion at *m/z* 197/199 with a moderate collision energy of ~30 eV (Figure 11).

In a final series of experiments, we exposed calf thymus DNA to a low flux of H_2O_2 (10 μM/h) and plasma levels of halides in the presence and absence of EPO and utilized stable isotope dilution LC/ESI/MS/MS analysis to determine whether Br-A was formed. Typical chromatograms of DNA hydrolysates analyzed by HPLC with on-line tandem mass spectrometry in the positive ion mode while monitoring the transitions of *m/z* 214 → 197 for BrA and *m/z* 224 → 206 for the $Br[^{13}C_5,^{15}N_5]A$ internal standard are shown in Figure

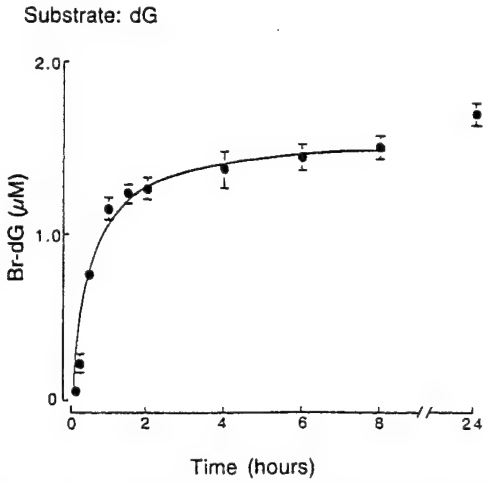

FIGURE 8: Time course study of bromo-dG formation by eosinophil peroxidase. dG (2 mM) was incubated with eosinophil peroxidase (57 nM), NaCl (100 mM), NaBr (100 μM), glucose (100 μg/mL), and glucose oxidase (20 ng/mL) in sodium phosphate buffer (20 mM, pH 7.0) at 37 °C. At the indicated times, excess methionine (10 mM) was added and the production of BrdG assessed by reverse phase HPLC at 254 nm as described in Experimental Procedures. Data represent the means ± SD of triplicate determinations for a representative experiment performed at least three independent times.

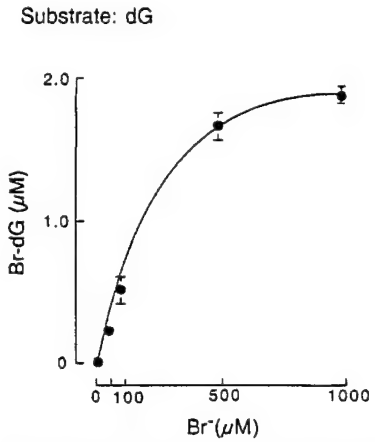

FIGURE 9: Bromide dependence of dG bromination by eosinophil peroxidase. dG (2 mM) was incubated with eosinophil peroxidase (57 nM), NaCl (100 mM), glucose (100 μg/mL), glucose oxidase (20 ng/mL), and the indicated concentrations of sodium bromide in sodium phosphate buffer (20 mM, pH 7.0) at 37 °C. Reactions were quenched by addition of excess methionine (10 mM), and the production of BrdG was assessed by reverse phase HPLC at 254 nm as described in Experimental Procedures. Data represent the means ± SD of triplicate determinations for a representative experiment performed at least three independent times.

12. A significant level of BrA was formed only in the presence of EPO (Figure 12).

DISCUSSION

The contribution of oxidative processes to carcinogenesis is now widely accepted (*1*−*10*). Much progress in this area involves use of stable markers of free radical reactions to identify specific chemical mechanisms of DNA damage in vivo. For example, hydroxylated, nitrated, aldehyde-modified, and chlorinated bases have been characterized and used to determine mechanisms of DNA, RNA, and nucleotide

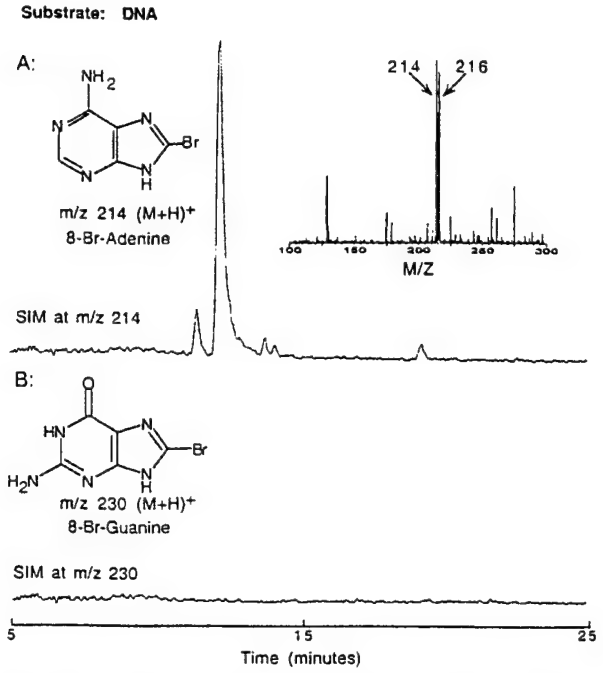

FIGURE 10: 8-Bromoadenine, but not 8-bromoguanine, is formed following exposure of DNA to the EPO-generated oxidant, HOBr. Calf thymus DNA (1 mg/mL) was incubated with HOBr (100 μM) in sodium phosphate buffer (20 mM, pH 7.0) for 60 min. The reaction was quenched by addition of excess methionine (10 mM), and then DNA was isolated, hydrolyzed, and analyzed by reverse phase HPLC with on-line ESI-MS analysis as described in Experimental Procedures. Production of BrA and BrG was assessed by analysis in the selected ion monitoring (SIM) mode at *m/z* 214 and 230, respectively. A major ion with a retention time identical to that of authentic 8-BrA, but not to that of 8-BrG, was observed. (Inset) In a separate HPLC run, the full scale mass spectrum of the ion eluting with *m/z* 214 and the retention time of 8-BrA was obtained. The identity of the DNA oxidation product (8-BrA) was further confirmed by noting the isotopic cluster expected for a monobrominated adenine adduct, with ions with a 1:1 M:M + 2 relative intensity at *m/z* 214 and 216.

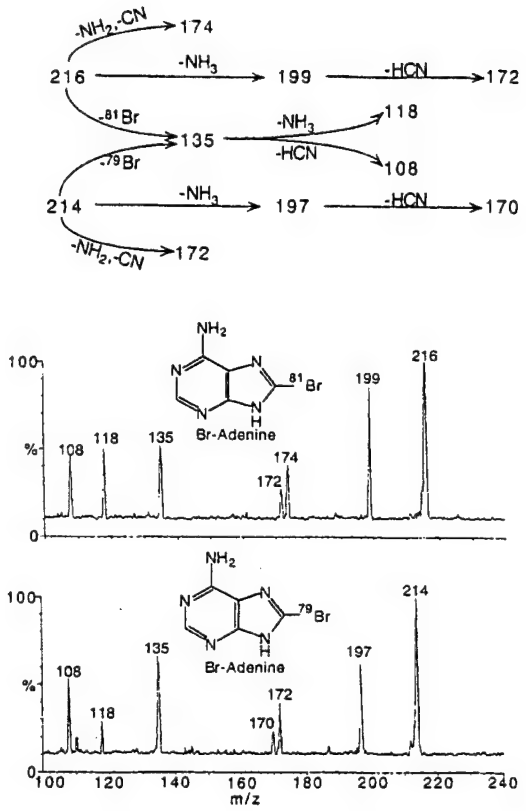

FIGURE 11: Positive ion electrospray ionization tandem mass spectrometric analysis of 8-bromoadenine. 8-BrG generated during HOBr-mediated oxidative modification of DNA (Figure 10) was analyzed by reverse phase HPLC with on-line electrospray ionization tandem mass spectrometry, as described in Experimental Procedures. (Top) Scheme for the proposed fragmentation patterns of protonated forms of ^{79}Br and ^{81}Br isotopomers of 8-bromoadeninine (*m/z* 214 and 216, respectively). (Middle) Daughter ion analysis of *m/z* 216 ($[M + H]^+$ for the ^{81}Br isotopomer) revealed the following ions: *m/z* 199 ($[M + H]^+ - NH_3$), 174 ($[M + H]^+ - NH_2 - CN$), 172 ($[M + H]^+ - NH_3 - HCN$), 135 ($[M + H]^+ - {}^{81}Br$), 118 ($[M + H]^+ - {}^{81}Br - NH_3$), and 108 ($[M + H]^+ - {}^{81}Br - HCN$). (Bottom) Daughter ion analysis of *m/z* 214 ($[M + H]^+$ for the ^{79}Br isotopomer) revealed the following ions: *m/z* 197 ($[M + H]^+ - NH_3$), 172 ($[M + H]^+ - NH_2 - CN$), 170 ($[M + H]^+ - NH_3 - HCN$), 135 ($[M + H]^+ - {}^{79}Br$), 118 ($[M + H]^+ - {}^{79}Br - NH_3$), and 108 ($[M + H]^+ - {}^{79}Br - HCN$).

damage in vitro and in vivo (*48*−*55*). However, the potential role of brominating oxidants in DNA damage and cancer development has not received much attention. The results of the studies presented here suggest that formation of reactive brominating species by the EPO/H_2O_2/Br^- system of eosinophils may be one pathway these cells contribute to oxidative modification of DNA and the nucleotide pool. Recent studies identify brominating oxidants as a distinct class of oxidants formed following eosinophil activation in vivo (*61*). Moreover, numerous cancers are notable for a significant eosinophilic infiltration in the cancerous tissues. The specific brominated bases that are identified may thus serve as markers for future studies aimed at determining the potential role of brominating oxidants in DNA oxidative damage in vivo.

On the basis of the results from the present report and recent published studies (*44*), we have generated a model of potential pathways through which brominating oxidants may contribute to oxidative modification of free bases, RNA, and DNA (Figure 13). Upon activation, the NADPH oxidase complex of eosinophils forms $O_2^{\bullet-}$, which both spontaneously and enzymatically dismutates to form H_2O_2. Concomitantly, eosinophil activation leads to the secretion of EPO into the extracellular compartment. In the presence of plasma levels of Br^-, EPO generates brominating oxidants such as HOBr, which can directly brominate free nucleosides and DNA forming stable monobrominated adducts (Figure 13). Identification of brominated adducts of each free base was confirmed by HPLC with on-line ESI-MS analysis. In the case of brominated purine bases, structural characterization as the 8-bromo-substituted analogues of adenine and guanine was further confirmed by NMR and tandem mass spectrometry. EPO-generated HOBr can also react with $O_2^{\bullet-}$ to form a $^{\bullet}$OH-like oxidant (eq 3) (Figure 13). The content of 8-hydroxyguanine, a marker of $^{\bullet}$OH-dependent DNA damage, was recently shown to significantly increase in DNA, RNA, and the nucleotide pool of cells exposed to a hypohalous acid generating system and enhanced intracellular $O_2^{\bullet-}$ (*44*).

One remarkable feature of these results is the finding that addition of a large molar excess of primary amines failed to

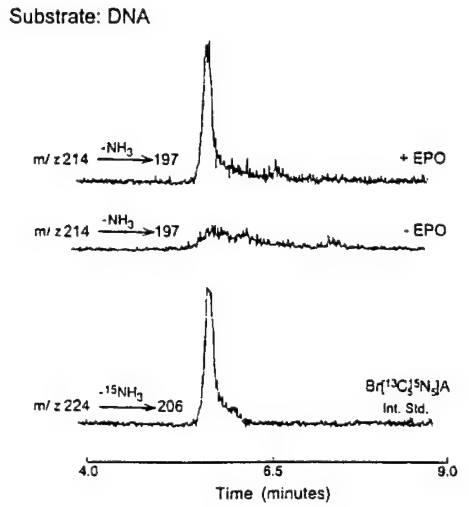

FIGURE 12: Positive ion electrospray ionization tandem mass spectrometric detection of 8-bromoadenine formed following DNA modification by the EPO/H_2O_2/Br^- system. Calf thymus DNA (1 mg/mL) was incubated overnight at 37 °C with a H_2O_2-generating system (10 μM/h; 100 μg/mL glucose and 20 ng/mL glucose oxidase) in Chelex-treated sodium phosphate buffer (20 mM, pH 7.0) supplemented with plasma levels of halides (100 mM NaCl and 100 μM NaBr) in either the presence (+EPO) or absence (−EPO) of EPO (105 nM). DNA was then isolated, heavy isotope-labeled internal standard (Br[$^{13}C_5$,$^{15}N_5$]A) added, and formation of 8-BrA assessed by HPLC with on-line tandem mass spectrometry using the multiple-reaction monitoring mode as described in Experimental Procedures. 8-BrA was detected while monitoring the transition between the molecular ion [*m/z* 214 ([M + H]$^+$ for the ^{79}Br isotopomer)] and the characteristic daughter ion at *m/z* 197 ([M + H]$^+$ − NH_3). The corresponding transition was monitored for the isotopically labeled internal standard.

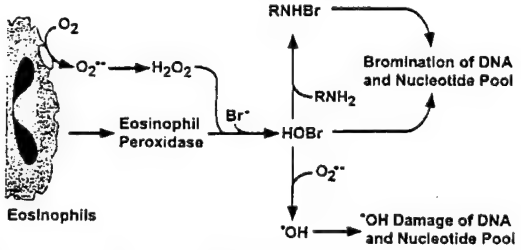

FIGURE 13: Model of potential pathways through which EPO-generated brominating oxidants may contribute to DNA oxidative damage.

block nucleoside bromination. These results are consistent with the finding that *N*-bromoamines serve as preferred brominating intermediates (Figure 7). Protein-bound and free primary amines are some of the most abundant nucleophilic moieties that reactive halogenating species will encounter in vivo. The ability of *N*-bromoamines to promote bromination of free nucleosides and adenine in DNA is thus anticipated to prolong the effective half-life of brominating agents formed in vivo and to help "funnel" brominating equivalents toward stable ring-brominated adducts of nucleosides (Figure 13). Similar results were observed for bromination of the aromatic amino acid tyrosine (*75*). In this respect, bromination reactions are distinct from results reported for the chlorination of the pyrimidine cytosine (*48*), the double bond of sterols (*76*), and phenolic targets such as tyrosine (*77*), all of which appear to be mediated by molecular chlorine, and not by *N*-chloroamines. Moreover,

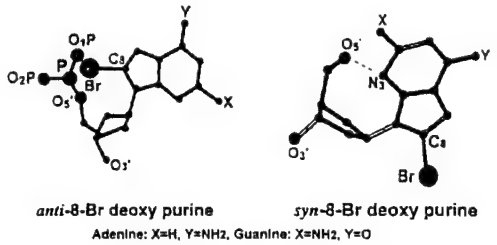

FIGURE 14: Structures of 8-bromopurines having anti and syn glycosyl torsions for the bases. 8-Bromopurines with the anti glycosyl conformation will result in short contacts between bromine and atoms of the sugar−phosphate backbone chain. The steric hindrance is avoided in 8-bromopurines having bases in the syn conformation, which is stabilized by an $O_{5'}$−H···N_3 hydrogen bond. The conditional demands for syn purine bases in double-helix DNA are not favorable for guanine, as opposed to adenine, as explained in the text.

whereas bromination of free nucleosides such as dG and dC occurs readily at neutral pH, an acidic environment is required for chlorination of all nucleosides (Z. Shen, W. Wu, and S. L. Hazen, unpublished observations; *48*). It has been suggested that the abundance of the amino acid taurine within phagocytes (~20 mM) may serve to trap oxidized forms of halides and protect critical intracellular targets from oxidation and halogenation (*40*). The facile reduction of *N*-bromoamines resulting in nucleotide halogenation reactions at neutral pH raises the interesting possibility that high intracellular levels of the β-amino acid may instead promote modification of nucleobases in the free nucleotide pool, as well as accessible nucleotide-containing polymers such as various RNAs or DNAs.

Another interesting result in this study is the observation that 8-bromoadenine is a major stable purine oxidation product formed following exposure of double-stranded DNA to the EPO/H_2O_2/Br^- system, while no detectable 8-bromo adduct for guanine in DNA was formed. These results are the reverse of the rank order for bromination observed with free nucleosides at neutral pH. Conformational analysis of the steric requirements for incorporation of a halide into the C-8 position of purine bases both in free nucleosides and within the major groove of the DNA double helix provides a rationale for these observations (Figure 14). Addition of a bulky bromo group at the C-8 position ortho to the glycosyl link, due to steric constraints, forces the purine bases to adopt a syn glycosyl conformation (Figure 14) (*79*). In contrast, a smaller hydroxyl group at the C-8 position of purines, such as in formation of 8-hydroxyguanine or 8-hydroxyadenine, is readily accommodated in the anti glycosyl conformation. While a syn conformation for purine bases is quite frequent in free nucleosides, a change in the glycosyl torsion from anti to syn in double-stranded DNA would result in a significant free energy expenditure due to changes in the base pairing scheme (below). For purine bases either free or in the C2′-endo sugar pucker (B-DNA), a syn glycosyl conformation results in an additional stability contributed by an intramolecular O5′−H···N3 hydrogen bond (*79*). Therefore, bromination at C-8 in the free purine bases or the nucleosides can occur readily.

In contrast, as noted above, for bromination to occur at the C-8 position of either adenine or guanine in a DNA double helix, the purine base must convert its glycosyl conformation from anti to syn. In both cases, this conversion

will necessitate the transformation of a Watson−Crick base pair to a Hoogsteen pair (*80*). A Hoogsteen A·T base pair, like its Watson−Crick pair, has two hydrogen bonds and can be formed without loss of stability. For a G·C Hoogsteen pair to be formed with the G in the syn conformation, the complementary cytosine base needs to be protonated (*79*). This will impose a significant free energy restriction by the demand of a lower pK_a for such a transition to occur. In addition, in the Hoogsteen geometry a G·C pair loses stability by having only two hydrogen bonds, in contrast to three hydrogen bonds for its Watson−Crick geometry (*80*). These restrictions readily explain the predominance of 8-BrA formation and absence of any 8-BrG adducts, following exposure of calf thymus DNA to either reagent HOBr or the EPO/H_2O_2/Br^- system.

One critical question that has not yet been resolved is whether bromination of purine and pyrimidine targets takes place in vivo. Clearly, the probability of a brominating oxidant diffusing through a gauntlet of cytosolic scavengers unscathed before reaching a nuclear DNA base as its ultimate target will be a low-probability event (as are all DNA oxidation events). However, it should also be recognized that the oxidation event does not have to take place inside the cell nucleus, but may occur either within the cytosol (i.e., the nucleotide pool) or even within the extracellular compartment. Parasitic infections are accompanied by increased cell death and lysis at the site of inflammation. Moreover, brominated bases can be taken up and incorporated into DNA and RNA of cultured mammalian cells (*78*). Though mammalian cells are equipped with numerous surveillance mechanisms for removal of modified bases from the nucleotide pool, the fidelity of these systems is not absolute (*20*). Indeed, exposing cultured cells to brominated bases in the medium results in sister chromatid exchanges and mutation (*78*). We have recently demonstrated that bromination of extracellular targets (protein tyrosine residues) by activated eosinophils occurs in vivo at sites of inflammation (*61*). The ability of certain free nucleobases to undergo bromination at neutral pH suggests that similar events may occur in vivo. Thus, in the setting of a chronic parasitic infection where decades of eosinophil-mediated inflammatory injury can occur, bromination of extracellular or cytosolic nucleobases may occur.

In summary, the results presented here suggest that specific brominated DNA bases may serve as novel and specific markers for monitoring oxidative damage of DNA and the nucleotide pool by brominating oxidants. Moreover, they identify adenine, rather than guanine, as a more likely target for halogenation of purine bases within double-helix DNA. The detection of brominated bases in eosinophil-rich inflammatory lesions or cancers would strongly suggest that brominating oxidants formed by these cells contribute to the development of DNA damage in these disorders.

ACKNOWLEDGMENT

Mass spectrometry experiments were performed at the Mass Spectrometry Resource of the Cleveland Clinic Foundation and the Cleveland Mass Spectrometry Core Facility. NMR experiments were performed in the Cleveland Center for Structural Biology.

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BI001961T

Eosinophil Peroxidase Brominates Free Nucleotides and Double Stranded DNA

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Chronic parasitic infections are a major risk factor for cancer development in many developing countries. Oxidative damage of DNA may provide a mechanism linking these processes. Eosinophil recruitment is a hallmark of parasitic infections and many cancers, and eosinophil peroxidase (EPO), a secreted hemoprotein, plays a central role in oxidant production by these cell. Here, we show that EPO effectively uses plasma levels of bromide as co-substrate to brominate nucleic acids (free and within double helix DNA) forming several stable novel brominated adducts. Products were characterized by HPLC with on-line UV spectroscopy and electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS). Ring assignments for brominated purine bases as their 8-bromo adducts were identified by NMR. Using stable isotope dilution LC/ESI/MS/MS we show that while guanine is the preferred purine targeted for bromination as a free nucleobase, 8-bromoadenine is the major stable purine oxidation product generated following exposure of double-stranded DNA to either HOBr or the EPO-H_2O_2-Br^- system. Bromination of nucleobases was inhibited by the thiol ether methionine, but not by large molar excesses of primary amines. *N*-mono-bromoamines were subsequently shown to be effective brominating agents for free nucleobases and adenine within intact DNA. A rationale for selective modification of adenines but not guanines in double-stranded DNA based upon stereochemical criteria is presented. These results identify specific brominated DNA bases that may serve as novel markers for monitoring oxidative damage of DNA and the nucleotide pool by brominating oxidants.

Shen, Z., and Hazen, S.L. "Eosinophil peroxidase brominates free nucleotides and double stranded DNA," Abstract 263, of 7th Annual Meeting of the Oxygen Society, Nov 16-20, 2000. *Free Radical Biology & Medicine*, (2000) Vol 29, Supplement I, S85.

Authors: Shen, Z., and Hazen, S.L.

Title: 200 character limit: 52

Oxidative Damage of DNA by Peroxidase-mediated Bromination

Introduction: 500 character limit: 488

Chronic parasitic infections are a major risk factor for cancer development in many underdeveloped countries. Oxidative damage of DNA may provide a mechanism linking these processes. Eosinophil recruitment is a hallmark of parasitic infections, and eosinophil peroxidase (EPO), a secreted hemoprotein, plays a central role in oxidant production by these cells. EPO is the only known human enzyme which generates brominating oxidants at plasma levels of halides. Here, we show that EPO brominates free and DNA-bound nucleobases forming several stable brominated adducts.

Methods and Instrumentation: 500 character limit: 495

Deoxyribonucleic acids (dA, dC, dG, dT) and DNA were each exposed to either EPO, H_2O_2 and plasma levels of halides, or the brominating oxidant, HOBr. Products were analyzed by HPLC with on-line UV spectroscopy and electrospray ionization tandem mass spectrometry (ESI/MS/MS) on a Micromass Quatro II triple quadrupole MS system. Ring-assignments for brominated adducts were confirmed by 1D NMR and 2D NOESY performed on a Varian Inova 500 MHz spectrometer. 8-bromo-[$^{15}N_5{}^{13}C_5$]-adenine was used for stable isotope dilution quantification in biological samples using LC/ESI/MS/MS.

Preliminary Data: 1500 character limit: 1298

Exposure of each deoxynucleotide to either HOBr or the EPO-H_2O_2-Br^- system resulted in formation of novel distinct monobrominated adducts of each base. Because of the acid stability of purines and the desire to develop a method for the detection and quantification of brominated bases in DNA hydrolysates from biological material, further structural studies focused on characterizing the brominated products formed with adenine and guanine. 8-Bromo-2'deoxyG (8-BrdG) and 8-bromo-2'deoxyA(8-BrdA) were identified as major products formed following exposure of dG and dA to EPO-generated HOBr by 1D NMR and 2D NOESY. Formation of 8-BrdG required the presence of EPO, H_2O_2 and Br^-. Addition of either methionine, a potent scavenger of reactive brominating species, or sodium azide (NaN_3), a peroxidase inhibitor, blocked 8-BrdG formation. A high yield of 8-BrdG was formed using physiological levels of Br^- (in the presence of plasma levels of Cl^-). Reactive brominating intermediates (N-bromoamines) were also found to readily promote aromatic bromination of DNA. Using stable isotope dilution LC/ESI/MS/MS we next demonstrated that adenine, and not guanine, was the major target for bromination in intact DNA. 8-Bromoadenine (8-BrA) was the major stable product formed following DNA modification by either reagent or EPO-generated brominating oxidants. Finally, using computer-assisted modeling we explain the apparent preference for bromination of adenine vs. guanine residues within the major fold of the DNA helix.

Novel Aspect: 100 character limit:100

LC/ESI/MS/MS detection of brominated DNA bases may serve as a valuable tool for monitoring DNA damage and cancer risk.

[Oral Presentation, June, 2000, American Society of Mass Spectrometry Meeting, Long Beach, CA.]